UNIVERSIDADE DE LISBOA
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DEPARTAMENTO DE BIOLOGIA

Impacts of forest fragmentation on small mammals in the southern Amazonia: A geometric morphometric approach

"Documento Definitivo"

Doutoramento em Biologia e Ecologia das Alterações Globais
Especialidade de Biologia e Ecologia Tropical

Welvis Felipe Fernandes Castilheiro

Tese orientada por:
Professora Doutora Maria da Luz Mathias
Doutora Sofia Isabel Vieira Gabriel
Professor Doutor Manoel dos Santos-Filho

Documento especialmente elaborado para a obtenção do grau de doutor

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Nota Prévia

Nos termos do nº1 do Artigo 44, Capitulo IV, do Regulamento de Estudos Pós- Graduados da Universidade de Lisboa, publicado no Diário da República, 2.ª série — N.º 155 — 11 de agosto de 2017, na elaboração desta tese foi efectuado o aproveitamento total de resultados de trabalhos a submeter para publicação em revistas internacionais com factor de impacto e com arbitragem científica (indexadas no ISI Web of Knowledge – Journal Citation Report®), os quais integram alguns dos capítulos da presente tese. Tendo em conta que os referidos trabalhos foram realizados em colaboração com outros autores, o candidato esclarece que em todos eles lideraram e participaram activamente na concepção, recolha dos dados e execução do trabalho experimental, bem como na análise e discussão dos resultados obtidos, bem como na escrita dos respectivos artigos científicos.

Apesar desta tese integrar artigos científicos a submeter para publicação, o padrão de formatação apresentado foi uniformizado para todos os capítulos.

Lisboa, junho de 2020

Welvis Felipe Fernandes Castilheiro

Para minha filha Serena,

Pelo fato da sua existência e de eu poder contar com esse apoio!!!

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Resumo

A perda e a fragmentação de habitats são há muito reconhecidas como importantes impulsionadoras da drástica redução da biodiversidade e da degradação dos ecossistemas. O conceito de fragmentação refere-se ao processo através do qual um habitat contínuo original, terrestre ou aquático, é dividido, como o próprio nome indica, em mosaicos/fragmentos, mais ou menos isolados. Fragmentos florestais são áreas de vegetação natural interrompidas por barreiras antrópicas (criadas por ação humana), capazes de diminuir significativamente o fluxo de organismos, sejam animais, pólen ou sementes. A divisão em partes de uma área antes contínua faz com estas adquiram condições ambientais muito diferentes.

No sul da Amazónia, a fragmentação de habitats constitui uma das principais causas de stresse ambiental para pequenos mamíferos. Para avaliar os impactos do processo de fragmentação florestal em populações de quatro espécies de pequenos mamíferos (*Proechimys longicaudatus*, *Necromys lasiurus*, *Marmosa demerarae* and *Monodelphis glirina*) foi usada uma abordagem tendo por base a morfometria geométrica. Para tal, foram avaliadas três aplicações distintas desta ferramenta na análise fenotípica da mandíbula das quatro espécies em estudo: i) a componente simétrica, que explica a variação entre os indivíduos, para avaliar a variação populacional no tamanho e na forma da estrutura mandibular entre fragmentos; ii) a componente assimétrica, avaliando os níveis de assimetria flutuante, ou seja, da variação intra-individual de estruturas com simetria bilateral, esquerda- direita, permitindo a avaliação dos níveis de perturbação durante o desenvolvimento embrionário; iii) e a integração morfológica e modularidade, avaliando a tendência de diferentes características variarem de forma conjunta e coordenada numa estrutura morfológica. É sabido que as alterações provocadas pela fragmentação de habitat têm reflexo nos níveis de abundância, estrutura populacional e ocupação do habitat de indivíduos que enfrentam tais mudanças no seu ambiente. Até que ponto é possível detetar alterações morfológicas ao nível individual e populacional é ainda pouco estudado, particularmente em espécies de pequenos mamíferos Neotropicais.

Neste contexto, o principal objectivo deste estudo consistiu na avaliação do impacto de fragmentos florestais de diferentes tamanhos sobre o fenótipo de quatro espécies de pequenos mamíferos através da análise das suas mandíbulas, utilizando uma abordagem com base na Morfometria Geométrica. A área de estudo abrange uma das regiões mais impactadas e desmatadas da floresta amazónica, resultando numa paisagem hiper-fragmentada cercada por pastagens e com algumas áreas altamente isoladas.

Assim, foram amostrados um total de 334 indivíduos adultos na área de estudo e utilizados para análise morfométrica: 143 amostras de espécimens pertencentes à ordem Rodentia (82 ratos espinhosos de cauda longa, *Proechimys longicaudatus*, e 61 ratos bolo de cauda peluda, *Necromys lasiurus*) e 191 espécimes pertentencentes à ordem Didelphimorphia (109 cuícas, *Marmosa demerarae*, e 82 cuícas da Amazónia, *Monodelphis glirina*). A área de estudo abrangida neste estudo localiza-se no sul da Amazónia, no município de Alta Floresta, no norte do estado de Mato Grosso, Brasil. A região era anteriormente coberta por floresta tropical mas atualmente está restrita a fragmentos de diferentes tamanhos cercados por uma matriz de pastagem maioritariamente aberta, abrangendo cerca de 40% da área do estado. A amostragem e a recolha de dados foram realizadas num total de dezassete fragmentos florestais e duas áreas contínuas. Os fragmentos foram agrupados de acordo com seu tamanho: dez fragmentos pequenos (S) variando entre 4 e 26 hectares e sete fragmentos grandes (L) entre 106 e 1763 hectares. Foram também amostradas duas áreas adjacentes de floresta contínua (C), ambas acima de 30 000 hectares.

Os resultados deste estudo revelaram que residir em fragmentos pequenos tem causado alterações significativas na forma das mandíbulas dos indivíduos na maioria das espécies analisadas. As distâncias morfométricas entre os grupos são sistematicamente e significativamente maiores quando a comparação é efetuada envolvendo fragmentos pequenos em comparação com fragmentos grandes ou áreas contínuas, quando disponíveis para comparação. Para as duas espécies de maiores dimensões (*Proechimys longicaudatus* e *Marmosa demerarae*), foram detetados desvios morfológicos significativamente maiores em relação à simetria bilateral de ambas as hemi-mandíbulas (assimetria flutuante) entre os indivíduos que habitam pequenos fragmentos florestais. Elevados níveis de assimetria flutuante refletem elevados níveis de stresse durante o desenvolvimento embrionário, neste caso associado ao stress ambiental inerente às alterações provocadas pela fragmentação do habitat. Por outro lado, os níveis elevados de isolamento entre fragmentos correlacionaram-se positivamente com elevados níveis de assimetria flutuante. Contudo, apesar de positiva, esta correlação não foi estatisticamente significativa para nenhuma das espécies. Per se, os elevados níveis de isolamento dos fragmentos não parecem ter causado impactos significativos a qualquer das espécies em estudo a este nível, possivelmente porque não será ainda de todo impossível a todos os indivíduos ultrapassarem a barreira geográfica imposta pela matriz de pastagem. A área do fragmento em que habitam parece ser o factor mais importante e determinante dos níveis de assimetria flutuante observados.

Por último, foi possível verificar que, em três das quatro espécies analisadas (*P. longicaudatus*, *N. lasiurus* e *M. glirina*), os animais residentes em pequenos fragmentos apresentaram alterações no padrão esperado de modularidade e integração morfológica da mandídula, o que pode possivelmente alterar a funcionalidade morfológica dos indivíduos, evidenciando uma disrupção nos padrões normais.

Tendo por base os resultados obtidos com as diferentes análises morfométricas das mandíbulas destas quatro espécies de pequenos mamíferos em estudo, é possível retirar algumas conclusões gerais com implicações para a conservação da biodiversidade desta região. Assim, com base em dados empíricos, reforça-se a necessidade de manutenção de fragmentos de grandes dimensões num contexto de fragmentação florestal, actuando como repositório dos níveis de diversidade morfológica o mais semelhantes à floresta contínua original possível. Da mesma forma, a manutenção de corredores ecológicos é importante, não apenas com árvores nativas, mas também com subcoberto arbustivo, criando fragmentos de conexão, permitindo que populações isoladas se tornem meta-populações conectadas entre fragmentos e áreas contínuas. Isso promoveria a redução destes impactos menos óbvios ao nível individual e populacional de natureza morfológica, consequentemente maximizando a persistência das próprias espécies. O grau de isolamento de fragmentos revelou ser uma métrica da paisagem tendencialmente importante numa dinâmica de fragmentação florestal; as espécies de pequenos mamíferos estudadas tendem a ter níveis mais altos de stresse no desenvolvimento quando os fragmentos são mais isolados. A dispersão dessas espécies é facilitada em paisagens com menor isolamento entre fragmentos, consequentemente menores níveis de alterações morfológicas tendem a ocorrer.

De uma forma geral, foi possível concluir que as diferenças morfológicas encontradas não se associaram com grupos taxonómicos específicos (neste caso roedores versus didelfídeos) mas sim com as características individuais e necessidades ecológicas das espécies em estudo. Neste estudo, à semelhança de outros, foi possível concluir que nem todas as espécies reagem ao processo de fragmentação da mesma forma e em simultâneo. Das quatro espécies analisadas, as de maiores dimensões, *M. demerare* e *P. longicaudatus*, revelaram níveis de assimetria flutuante significativamente maiores em fragmentos pequenos como resposta a maiores níveis de stresse ambiental durante o desenvolvimento embrionário. No entanto, todas espécies em estudo apresentaram variações fenotípicas significativas associadas à redução de habitat observada nos fragmento pequenos.

Outro aspecto relevante é a importância da base de dados que originou este trabalho. Os resultados aqui apresentados foram obtidos a partir de animais amostrados em 2009 (numa área sujeita a fragmentação há aproximadamente 55 anos). Apesar de ter decorrido um período relativamente curto relativamente à perda de área de habitat florestal, foi possível detetar alterações morfológicas nos animais como consequência da fragmentação florestal. Com este estudo, em contraste com a maioria dos estudos avaliando mudanças no tamanho efetivo da população ou perda de diversidade genética, foram obtidas informações valiosas sobre as consequências geralmente ocultas desses processos. Estes dados podem constituir uma linha de base de referência para comparação com estudos futuros de longo prazo, a partir de um período referente às primeiras cinco décadas de fragmentação da floresta amazónica. Como importantes características para a conservação é sugerida uma organização da paisagem contendo grandes Unidades de Conservação interconectadas por corredores florestais e inseridas numa matriz de sistemas agroflorestais, talvez uma utopia na conjetura atual. Mas pode ser a melhor alternativa para a conservação da biodiversidade e processos ecológicos, especialmente se combinada com ações de educação ambiental que buscam incluir comunidades locais em programas de conservação. Aqui, confirmamos que as espécies estudadas respondem diferentemente à perda e fragmentação de habitat. Assim, estudos futuros não devem fazer generalizações para determinado grupo de espécies uma vez que espécies taxonomicamente próximas podem responder de maneira diferencial às mudanças no ambiente. É, portanto, essencial entender os múltiplos factores determinantes para a maior ou menor resiliência das populações que constituem a comunidade de pequenos mamíferos nestas paisagens fragmentadas. Além disso, os esforços devem ser concentrados na construção de modelos gerais que considerem respostas diferentes das espécies.

Palavras-chave: Amazónia, pequenos mamíferos, morfometria geométrica, mandíbula, alterações morfológicas.

Abstract

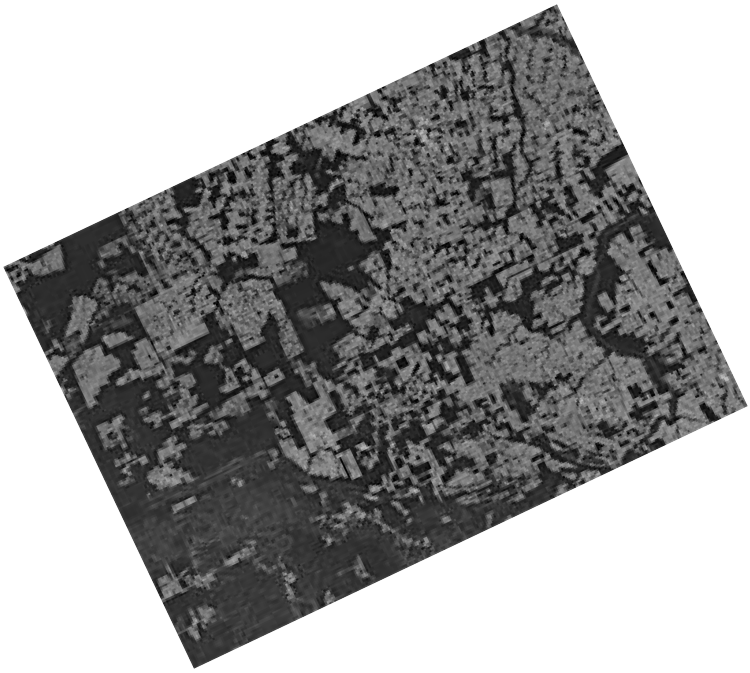
Habitat loss and fragmentation have long been recognized as major drivers of biodiversity depletion. In Amazonia, forest fragmentation constitutes a major cause of environmental stress for many organisms. Here, we addressed the effects of differential fragment sizes on the mandible phenotype of four species of small mammals (*Proechymis longicaudatus*, *Necromys lasiurus*, *Micoureus demerarae* and *Monodelphis glirina*) using a geometric morphometry approach. This technique allowed to address: i) the symmetrical component, assessing population variation in shape and size; ii) fluctuating asymmetry, inferring disturbance during embryonic development; iii) morphological integration and modularity, allowing to identify deviations in the mandible's morphological structure.

The study area encompasses a hyper-fragmented area in southern Amazonia, characterised by forest landscape surrounded by pasture, with some highly isolated patches. Sampling and data collection were performed in 17 forest fragments grouped according to size: ten small fragments (4-26 hectares), seven large fragments (106-1763 hectares), and two surrounding areas of continuous forest (C≥30,000 hectares). Our results reveal that morphometric distances between groups of animals inhabiting different sized fragments are significantly higher when pairwise comparison involve small fragments. Fluctuating asymmetry was also significantly higher in small fragments but only for both species of larger body size (long- tailed spiny rat, *P. longicaudatus,* and woolly mouse opossum, *M. demerarae*). More isolated fragments were positively correlated with higher fluctuating asymmetry levels, although not significantly. Finally, in small fragments, changes in the expected pattern of modularity and morphological integration were also detected for all but one study species (*M. demerarae*), with putative consequences for the structure and morphological functionality of individuals. Based on empirical morphological data, we highlight the need for the maintenance of large forest patches as repositories of somewhat similar levels of morphological diversity and functionality in contrast with small fragments, where most deleterious effects were detected.

Key Words: Amazonia, small mammals, mandible, morphological alterations, geometric morphometrics.

1. Chapter 1

1.1. General Introduction



1.1.1. Amazon rainforest and the expansion of the arc of deforestation

The Amazon, holding roughly one-third of the world's tropical rainforests, plays a vital role in biodiversity conservation, regional hydrology, climate and terrestrial carbon storage and provides shelter and food for a wide variety of animals and people (Posey 1985, Cerri et al. 2007, Laurance et al. 2002). However, the Amazon rainforest has the highest rate of deforestation in the world: 662,400 ha in 2017 (Cardoso et al. 2020). Accelerated deforestation has some causes: i) the urbanisation process of the forest's non-indigenous populations has increased tenfold since 1960 (Martins et al. 2016), reaching approximately 24 million people today; ii) the lack of family planning of people from other parts of Brazil; in addition to the increase in the resident population; iii) the exponential increase of soybean plantations occupying large territories, as well as the annual increase of logging and mining activities (both legal and illegal); iv) the expansion of road networks that increases access to forests for large farmers and entrepreneurs (Kirby et al. 2006); and v) fires set intentionally for human purpose (Pivello 2011). Therefore, in the southern Amazon region, what remains is an extremely fragmented forest (Barni et al. 2009).

Spatial patterns of forest loss are changing. Deforestation was originally concentrated from southeastern Pará through Mato Grosso, but new highways, roads, logging projects and human colonisation now penetrate deep into the central Amazonian forest. This region still has high deforestation rates, with the agricultural frontier advancing towards the forest: 500,000 km² of land extends from the southeast of Pará to the west, through Mato Grosso, Rondônia and Acre (Barni et al. 2009). This area was called the 'arc of deforestation of the Amazon'. Today, the deforestation arc extends into the middle of the forest (Barni et al. 2009, Fearnside 2016 see Figure 1).

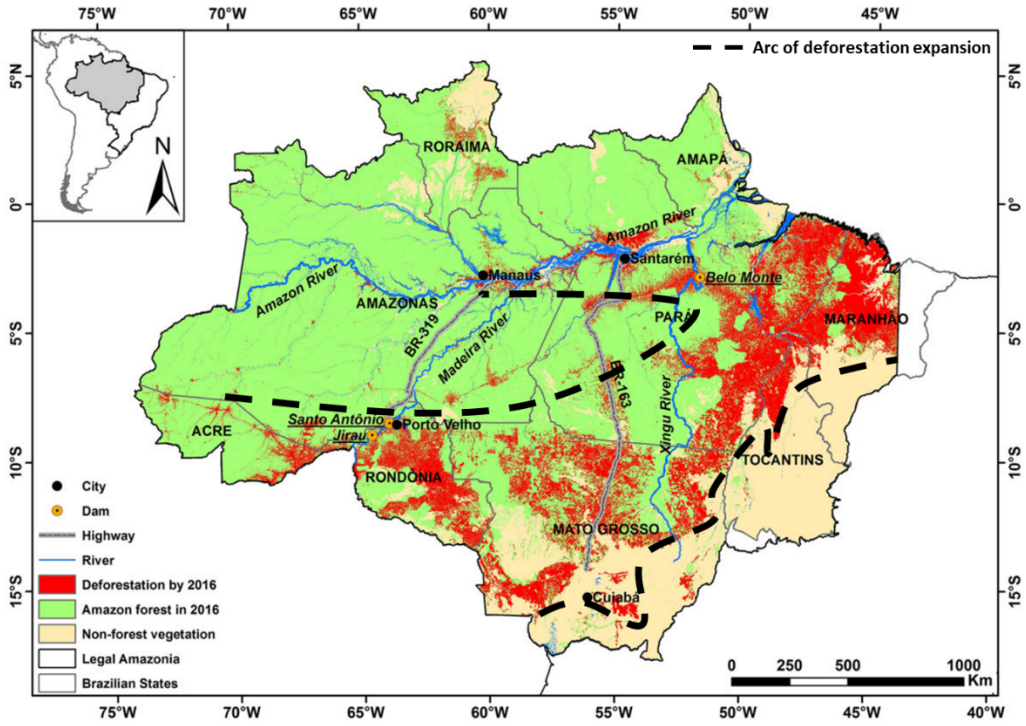


Figure 1 – Arc of Deforestation expansion in Amazon rainforest. Adapted from Fearnside 2016.

1.1.1. The problem: forest fragmentation

Fragmentation refers to changes in an original habitat, terrestrial or aquatic (van den Berg et al. 2001, Haddad et al. 2015); it is a process in which anthropic action divides a continuous habitat into isolated pieces, or fragments (Fahrig 2003). The fragmentation process has similar impacts on populations of resident species, described below.

The relationship between an increase in species richness and an increase in the sample area is widely debated (Dixo and Metzger 2009). Three proposals are referred to explain this relationship:

1. The species diversity recorded in a site is a consequence of the sampling effort: the larger the sample size, the larger the number of species (Laurance and Vasconcelos 2009).

2. The correlation between an area and its habitat diversity translates the relationship between the area and its biodiversity. Habitat diversity in an area increases as its size

increases, and because each habitat has a unique group of associated species, the area's species richness increases overall (Zimmerman and Bierregaard 1986).

3. The relationship between area and species within fragments is the result of a dynamic balance among resident populations and a balance between immigration and extinction, which is maintained by the relation between isolation degree and the connectivity of fragments (MacArthur and Wilson 1967).

1.1.2. Matrix, Isolation and Fragments′ shape

The mosaic of artificial habitats surrounding fragmented forest is called 'matrix'. Some examples in the Amazon include regenerating forests, farmland (e.g. soybean plantations) and, especially, pastureland (Laurance et al. 2011).

The type of matrix determines whether its effects will have a greater or lesser impact (Laurance et al. 2007). For example, fragments with regenerating forests in their surroundings have less arid microclimates and lower tree mortality than fragments surrounded by pasture or soybean plantations.

Matrices, primarily those of regenerating forests, can represent the ideal fragment connectivity and increase the richness of some populations surrounding the fragments (Barbosa et al. 2017). This connectivity is important for species survival as a greater degree of linkage between existing populations in forest remnants prevents extinction by allowing a given declining population to receive immigrants from other populations. Small mammals that live in connected populations, or 'metapopulations', are one example of the multiple taxa facing these challenging environmental conditions (Krohne 1997, Estavillo et al. 2013). Matrices that differ substantially from the forest vegetation and fragments that possess a high degree of isolation can both affect species richness (Gascon et al. 1999).

The degree of isolation may be a barrier to many species that use the fragment's core area (i.e. they are more dependent on the fragment's nucleus). The impediment for a species to migrate due to the absence of connections between fragments often results in genetic erosion within populations (Nemésio and Silveira 2010). The extinction of resident populations within completely isolated fragments is common. Recolonisation – one way for a species to re-occupy a fragment – would require sufficient proximity to potential source areas (Haddad et al. 2015).

Fragment shape is another important variable that explains the population dynamics within fragments (Ewers and Didham 2007). The more circular and dense a forest fragment

is, the greater its interior area in relation to the edge, which results in damage to a smaller portion of the forest. However, narrow or irregular fragments have a large interior-to-edge ratio, which means greater impact to the larger and more core-dependent species (Forman 1995).

The variables mentioned above are those best explaining population dynamics within fragments. However, for some populations and species, fragmentation may also pose morphological and genetic alterations, which can be more or less severe depending on the species' resilience (e.g. species of small mammals that respond differently to the effects of forest fragmentation - Teixeira et al. 2006, Santos-Filho et al. 2016, Fietz et al. 2014).

1.1.3. Study of small mammals in the Amazon

In this work, we studied two species of the order Rodentia – the long-tailed spiny rat (*Proechimys longicaudatus*, Rengger 1830) and the hairy-tailed bolo mouse (*Necromys lasiurus*, Lund 1840) – and two species of the order Didelphimorphia – the woolly mouse opossum (*Marmosa demerarae*, Thomas 1905) and the amazonian red-sided opossum (*Monodelphis glirina*, Wagner 1842) (see Figure 2).

Rodents comprise the largest order, Rodentia (Bowdich 1821), of the class Mammalia (Linnaaeus 1758), (Huchon et al. 2002, Stefoff 2008), including 35 families, 350 genera and approximately 6,400 species and subspecies (Mittwoch 1967).

Rodents are omnivores, having a preference not only for plant species but also for worms, arthropods and molluscs (Landry 1970). According to the predominant diet, the shape of a rodent's stomach varies, being either a simple sac or a complex structure (Ghoshal and Bal 1989). Some species are endowed with pockets in the oral cavity in which they store food to be slowly swallowed later (Mustapha et al. 2015).

Given the wide variety of ecosystems to which rodents have adapted, their morphological traits differ significantly from one family to another, mainly primarily due to the development of locomotion specialisations (Arregoitia et al. 2017). An important characteristic of rodents is their high reproductive capacity, which is largely responsible for their evolutionary success. This trait compensates for the high mortality rate among their populations, which are commonly preyed upon by reptiles, birds and other mammals (Francisco et al. 1995, Figueroa and Corales 2002, Schulte-Hostedde 2008, Ramnanan et al. 2016, Yánez-Muñoz et al. 2017). It is common for females to have several large litters per year (Francisco et al. 1995).

The long-tailed spiny rat (*P. longicaudatus;* Rengger 1830) is a rodent belonging to the family Echimyidae (Gray 1825). This species has a distribution covering southern Bolivia, northern Paraguay and central Brazil (Woods and Kilpatrick 2005). It is characterised by its ground foraging habits (Vieira and Diniz-Filho 2000), a diet with a tendency towards frugivory (Carvajal and Adler 2008) and year-round reproduction. Information on density and home range in forest fragments is lacking.

According to the International Union for Conservation of Nature (IUCN), rapid habitat conversion may threaten this species in the near future. Currently, the IUCN lists *P. longicaudatus* as 'least concern', considering its wide distribution, partial tolerance to habitat modification, presumed large population and slow rate of decline (Patton et al. 2016), with few studies addressing the species association to forest fragments (Santos-Filho et al. 2008, 2012a,b, 2016, Cáceres et al. 2010).

The distribution of the hairy-tailed bolo mouse (*N. lasiurus*, Lund 1840), a rodent belonging to the family Cricetidae (Fischer 1817), extends from central Brazil (south of the Amazon River) to extreme southeast Peru and northeast Argentina (Musser and Carleton 2005). This species also occurs in Paraguay and Bolivia. It has ground-dwelling habits (Pires et al. 2010) and a tendency towards a granivorous diet (Francisco et al. 1995, Magnusson et al. 1995). Mesquita and Passamani (2012) refer that *N. lasiurus* is abundant in corridor environments, indicating that corridors may be used to facilitate movement between forest fragments. However, the authors have not evaluated the impact of such fragmentation in the species dynamics.

The order Didelphimorphia (Gill 1872) comprises most mammals in the Americas, predominantly those that inhabit the forests of South America. This order is considered threatened by the fragmentation process by multiple assessments (Emmons and Feer 1997, Laurance and Bierregaard 1997, Nowak 1999). Within this order, the family Didelphidae alone (Gray 1821), which includes 19 genera and 95 species, constituting most marsupials, both living and extinct (Gardner 2008).

Didelphids have nocturnal habits and are generalists: they may be insectivorous, frugivorous, carnivorous or omnivorous, and their diet vary throughout the year based on age and reproductive status (Nowak 1999, Vieira and Astúa 2003, Casella and Cáceres 2006, Castilheiro and Santos-Filho 2013). These small mammals have five digits on each foot, with many of the species having an opposable hallux and pollex. They occupy and are adapted to a

great diversity of niches: arboreal, scansorial, terrestrial and semi-aquatic (Dalloz et al. 2012, Goin et al. 2016).

The majority of Didelphids are sexually dimorphic (males are larger than females) and polygamous. They become sexually active between 6 and 10 months of age, bear litters of 1 to 4 offspring per year and have a short lifespan of 1 to 3 years (Nowak 1999, Smith 2006, Astúa 2010). Their tails are mostly long and prehensile, but some have short, hairy tails (Rupert et al. 2014). Didelphids have conical upper incisors, polyprotodont lower incisors and large canines (Berkovitz 1967).

The woolly mouse opossum (*M. demerarae*, Thomas 1905), belonging the family Didelphidae (Gray 1821), features an extensive distribution ranging from Peru, Bolivia, Colombia and Venezuela, through Guyana and south to northern and central Brazil (Gardner 2007, Alberico et al. 2000). *M. demerarae* exhibits arboreal habits (Brito & Fernandez 2002), has a diet with a tendency towards insectivory (Fernandes et al. 2006, Santos-Filho et al. 2017) and reproduces only during the rainy season (Nowak 1999).

Figure 2. Species of small mammals studied in the southern Amazonia. Note: Images are not scaled.

Gardner (2007) reports that *M. demerarae* were captured in trees 50% of the time and that when released, escaped via trees or vines 85% of the time. Gardner estimated that densities

are as high as one per hectare and determined the home range of females to be roughly 0.10 ha, approximating that of other small didelphids in the forest area. However, Pires and Fernandez (1999) and Quental (2001) reported that males have a broader home range and can, therefore, overlap different populations in small fragments of Atlantic Coastal Forest in Brazil, concluding that males are less sedentary and territorial than females.

The woolly mouse opossum has already been studied extensively in relation to community structure and composition in fragmented landscapes: the species can persist in small forest fragments isolated for less than 10 years. Furthermore, the males, although arboreal, can cross areas of open vegetation from 150–350 m wide near the continuous forest (Fernandez et al. 2006). However, they are most often found in larger areas of forest and rarely in the matrix (Fonseca and Robinson 1990, Fernandez et al. 2006). Pires and Fernandez (1999) suggest that species in forest fragments persists in metapopulations. Therefore, migration by males would increase gene flow and facilitate species persistence and survival.

The amazonian red-sided opossum (*Monodelphis glirina,* Wagner 1842), belongs to the family Didelphidae (Gray 1821), is primarily terrestrial, with an omnivorous diet that includes insects (Nowak 1999, Emmons and Feer 1997, Castilheiro and Santos-Filho 2013). The home range of *M. glirina* is 0.12–0.18 ha in forest fragments (Macrini 2004), its distribution extends from the state of Pará in northeastern Brazil, south of the Amazon river, and through southern Perú and northern Bolivia (Anderson 1997).

Castilheiro and Santos-Filho (2013) reported that the diet of *M. glirina* in forest fragments is influenced by the 'edge effect', considering the greater number of insects present due to the cattle faeces in pastureland (matrix) occurring at the fragments' edges. In small fragments, *M. glirina* exhibit a higher consumption of these insects which can be explained by the larger border extension, which would facilitate the entry of beetles into forest areas.

The literature mentioned above discusses the negative impact of forest fragmentation on populations of small mammals in the Amazon. The majority of these studies have focused on small mammal distribution, richness, abundance, community composition and diet. In this study, a geometric morphometric approach was used as a tool to assess the effects of fragmentation on morphological changes at the species / populational / individual level.

1.1.4. Geometric morphometrics

Geometric morphometrics is defined as the statistical study of form variation in relation to causal factors (Klingenberg 2015). Geometric morphometrics differs from the traditional approach by employing not only linear measures such as lengths, widths, angles or proportions but also the robust statistical analysis of form (Marcus 1990, Klingenberg 2015). A set of methods were used to analyse variables that preserve all the geometric information contained in the original data based on Cartesian coordinates, such as anatomical points or landmarks (Slice 2005).

Landmarks can be classified into anatomical landmarks, mathematical landmarks and pseudo-landmarks (Dryden and Mardia 1998), having a basic premise of being homologous. Landmarks' coordinates have their relative positions as important geometric information, which is necessary to be submitted to superimposition, a popular technique to separate scaling, position and orientation differences from true differences in shape (Strauss and Bookstein 1982). In this way, coordinates' data is transformed into variables of size and shape. One of the key aspects of using geometric morphometrics is the equation 'form = shape + size', in which shape represents all the remaining geometric information when the effects of position, rotation and size are eliminated from an object (Klingenberg 2016).

Centroid size is defined as the distance from a landmark to the centroid, or centre of the shape's gravity (Neha 2015). This size measure guarantees the statistical independence between size and shape, which indicates the absence of allometrics (Klingenberg 2016). For two-dimensional studies, scanners and photographic cameras are the most used methods for obtaining the digitised images.

Photos are standardised, reducing or avoiding the introduction of variations that do not result from the biological processes being studied. Illumination tests must also be performed in search of better adaptations (Arnqvist and Martensson 1998, Muñoz-Muñoz et al. 2006, Muñoz-Muñoz et al. 2011, Franchini et al. 2016).

The camera must be fixed to prevent changes in the focal length from the camera to the object, and a scale bar must be included (Loy and Slice 2010).

To perform the placement and organization of landmarks into images, software such as TpsUtil and TpsDig are used (Rohlf 2006). The chosen landmarks must cover the entire shape of the object under study (Webster and Sheets 2010, Walker 2000).

Repeatability tests (i.e. the ability of an operator to digitise the same landmarks) should be performed using an analysis of variance while avoiding excessive variance (Strauss et al.

2003). Size and shape data are generated through the superposition of the Cartesian coordinates of each landmark. This process is the objective of the generalised Procrustes analysis (GPA): to remove translation, size and rotation effects (Gower 1975). Following GPA, differences in shape are described by differences (residues) in the coordinates of compatible landmarks between objects. The analysis generates a curved space called a non- Euclidean Kendall shape space (Kendall 1984).

One outcome of this analysis is the separation of the symmetric component (which looks for differences in shape between individuals) of the asymmetric component (which looks for differences in shape within the same individual), with values of morphological distances (Procrustes or Mahalanobis). These values can be organized by population groups, or sites within the study areas. Additional analyses, from exploratory, classificatory, tests of univariate, multivariate hypotheses and cluster analyses, to *a posteriori* analysis can be performed.

Exploratory analyses can be performed, such as the principal component analysis (PCA), which transforms the correlated variables into a new set of uncorrelated variables: the principal components (Monteiro and Reis 1999). When verifying the lack of digitising errors and the trends of variation in the shape, the hypotheses suggested can be tested (Fontaneto et al. 2004).

Analysis of Variance (ANOVA) can be used to infer differences between *n* groups by using the distances of distribution compared to the F-distribution and the significance obtained by permutation tests (Kao and Green 2008). The size variable can be analysed independently of the shape using the centroid size to perform the same type of analysis (Zelditch et al. 2004, Fernandes et al. 2009). For the hypothesis test using the shape, the indicated method is the Multivariate Analysis of Variance (MANOVA). The differences in shape can be visualised graphically through diagrams based on the PCA scores. Discriminant function analysis (DFA) and Canonical Variate Analysis (CVA) connect the configuration of landmarks, allowing the identification of the exact points of changes in shape within the structures (Fornel et al. 2010). Group analysis can be used to generate diagrams of morphological similarity between groups through Mahalanobis distances. The neighbour- joining (NJ) method can also be used to compare morphometric distance matrices with matrices of distances genetic or geographical distance. The Mantel test is used most often for testing these correlations (Mantel 1967).

MorphoJ (Klingenberg 2011) and R software (R Development Core Team 2011) are the most advised and currently used tools for performing the analyses mentioned above.

Quantification of shape can be related to the variables of allometry, sexual dimorphism and geographic variation as well as the functional and biomechanical aspects of biological shapes.

Allometry refers to differences in shape correlated with changes in the size of the object under investigation (Klingenberg 2016). Bou et al. (1987) investigated the relationships between allometry and locomotor adaptations in insectivores and rodents in long limb bones. The authors stated that allometric relationships of the different locomotor patterns are better reflected in insectivores and rodents than in other groups of mammals.

Landmark based geometric morphometrics is currently the most used tool in sexual dimorphism studies (Zelditch et al. 2004; Gidaszewski et al. 2009). Robinson et al. (1986) showed that male rats have a more elongated pre-optic nucleus area than female rats, which, through the geometric morphometrics technique, indicates a sex-dependent shape. Astúa (2010, 2015) also used geometric morphometrics to test sexual dimorphism in the skull shape of New World marsupials.

Bol'shakov et al. (2013) used geometric morphometrics to reveal coupled geographic variation in the mandible shape in two sympatric rodent species. Damasceno and Astúa (2016) also used this tool to evaluate patterns of geographic variation in the didelphid *Chironectes minimus*, the only semi-aquatic didelphid. The authors demonstrated how the animals of the Amazon differ from others, likely due to the existence of geographical barriers.

Fluctuating asymmetry (FA) affects bilateral structures due to embryonic developmental instability, during which subtle random deviations from symmetry occur (Leamy and Klingenberg 2005). Assessing FA as an individual trait variation of left-right differences allows for evaluation of the disturbance levels during the development of characteristics that affect an organism's ability to develop uniform traits (Palmer and Strobeck 1986, Klingenberg 2015). Yalkovskaya et al. (2016) analysed bank voles (*Myodes glareolus*, Schreber 1780) from the zones affected by pollution from three copper smelters in the Urals. The authors revealed a gradient effect of toxic exposure on FA levels of rodent cranial structures: an increase in FA under increasing technogenic impact is clearly manifested along local pollution gradients. Using the didelphid *Didelphis albiventris* as a model species, Teixeira et al. (2006) crossed data on fluctuating asymmetry with geographical information

systems data relating to environmental composition. The authors showed that environments that are more severely impacted resulted in statistically higher levels of FA.

Morphological integration and modularity are inherent and essential features of complex organisms (Klingenberg 2014). Integration typically manifests through the non-existence of modularity in the structure and development of an organism, which allows for adaptive modifications in some parts without interfering with the functionality of the others (Klingenberg 2014). Many methods are now available to study integration and modularity, all of which involve analysis of patterns found in trait correlation or covariance matrices. A detailed review of these methods can be found in Goswami and Polly (2010).

1.2. Aims and thesis structure

1.2.1. Aims

This thesis focused on the effects of forest fragmentation on the mandible phenotype of two rodent species (*Proechimys longicaudatus* and *Necromys lasiurus)* - and two didelphid species (*Marmosa demerarae* and *Monodelphis glirina*) inhabiting southern Amazonia using geometric morphometrics as a tool to investigate the following:

- ✓ mandibular morphometric variation in the study species occurring in continuous forest and different sized forest fragments,
- ✓ sexual dimorphism in mandibular morphometric traits between species and among forest fragments,
- ✓ the impacts of forest fragmentation on the patterns of fluctuating asymmetry (FA) in the study species,
- ✓ the potential of using fluctuating asymmetry as a biomonitoring tool in conservation biology of small mammals inhabiting fragmented habitats,
- ✓ the existence of modularity and morphologic integration pattern*s* on the mandibular modules in species of small mammals inhabiting a scenario of extreme environmental variation.

1.2.2. Thesis structure

This thesis is composed of five chapters:

- ✓ Chapter 1 includes the ‘General Introduction’, where the research topic and key concepts are presented.
- ✓ Chapter 2 focus on the ‘Effects of forest fragmentation on phenotypic variations of small mammals in southern Amazonia`.
- ✓ Chapter 3 analyses the ‘Fragmentation impacts on the fluctuating asymmetry of small mammals in southern Amazonia`.
- ✓ Chapter 4 reports the ‘Effect of forest fragmentation on mandibular modularity and morphological integration of Neotropical small mammals in southern Amazonia`.
- ✓ Chapter 5 includes the ‘Final Considerations, where the main findings presented in the previous chapters are summarised.

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2. Chapter 2

2.2. Effects of forest fragmentation on phenotypic variations of small mammals in southern Amazonia*

*** Data in this chapter will be submitted as a paper to Landscape Ecology**

Effects of forest fragmentation on phenotypic variations of small mammals in southern Amazonia

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Abstract

Context. Forest fragmentation refers to the partition of a continuous habitat into several fragments separated by matrix habitat. Consequences for Amazonian small mammals on the morphological variation under this dynamic are unknown and need to be clarified.

Objectives. We intend to evaluate mandibular morphometric variation between different sized forest fragments and, when possible, continuous forest of two species of order Rodentia and two species of order Didelphimorphia inhabiting southern Amazonia.

Methods. Specimens were collected from 17 forest fragments, classified as small or large according to their area, and two continuous areas. A total of 334 adult individuals were sampled for morphometric analysis. We symmetrized landmarks on both sides of each hemi- mandible and analysed both size and shape variation.

Results. Both rodents' species did not exhibit significant sexual dimorphism in mandible size but in both didelphids males were significantly larger than females. Also, regarding mandible shape, significant differences between sexes were observed for both didelphid species. Size showed a significant effect on shape for both orders. Mandible shape was significantly different between fragment groups for most comparisons in both orders.

Conclusions. Our results suggest that, regarding mandible shape, morphometric distances of small mammal populations between fragments groups is always significant higher when comparing small fragments with another group.

Considering the observed morphometric changes resultant of forest fragmentation, evaluation of conservation target species needs to enlarge selection criteria and not exclusively rely on species richness and abundance or even genetic diversity loss.

Keywords: Neotropical small mammals, mandible, sexual dimorphism, symmetry, allometry

2.2.1. Introduction

The Amazonian region has the largest rainforest in the world, covering approximately one-third of South America, with a total area of 5,500,000 km^2. It harbours nearly one third of the world's known species and is one of the planet's climate regulation 'hot spots' (Heckenberger et al. 2007). Thus, the preservation of the Amazonian forest is critical to the present global scenario of environmental and climate change (Brondízio et al. 2016; Nobre et al. 2016). However, increasing exploitation of natural resources and political influence favouring agrobusinesses has accelerated urbanization and land conversion for agriculture, leading to the destruction of large areas and the fragmentation of the rainforest (Michalski et al. 2008; Pereira et al. 2020).

Forest fragmentation is a major environmental issue and a global concern. It refers to the partition of a continuous habitat into several fragments of different sizes, differentially isolated from each other, surrounded by anthropogenic landscape (Ezcurra 2016). The consequent habitat loss and reduction of ecological connectivity among populations caused by dispersal inhibition or disruption can ultimately favour inbreeding depression and potentially local extinction (e.g. Coulon et al. 2004; Banks et al. 2005b; Bergl and Vigilant 2007; Fietz et al. 2014).

Morphological effects of forest fragmentation can be expected given the marked changes in habitat size and quality, both in terms of resource availability and disturbance regimes experienced by individuals (Saunders et al. 1991; Fischer and Lindenmayer 2007). Habitat fragmentation makes migration between disconnected populations difficult. These small isolated populations are vulnerable to genetic diversity loss, suffering an extinction risk due to decreased migration rates and genetic flow between populations. In this way, forest fragmentation can affect the genotype. But also, the phenotype for promoting adaptive

divergence in case of diverging natural selection (Hermes et al. 2016). However, different species may be differently affected, depending on their resilience to the altered conditions of a fragmented landscape. Some individual and ecological traits seem to determine a greater resilience towards fragmentation perturbations, such as a smaller body size, larger home range, greater dispersion ability, lower dependence of trees, less territoriality, higher abundance or a generalist diet (Gladstone-Gallagher et al. 2019). Such relationships are partly present in a variety of taxa such as frogs (Tocher et al. 1997), some species of bats (Kalko 1998), ground-foraging birds (Vasconcelos 1999) and small mammals (Rocha et al. 2011).

Amazonian rodents and didelphids are amongst the most diverse mammals in South America, with about 280 species described (~40% of all mammals) (Paglia et al. 2012). Species of both groups play important roles as ecological services' providers in neotropical ecosystems, not only as dispersers of seeds and mycorrhizal fungi (Vieira et al. 2006, Horn et al. 2008) but also as predators of seeds, invertebrates, small vertebrates and eggs (Cáceres and Monteiro-Filho 2001, Vieira et al. 2011) or as preys to larger mammals, birds and snakes (Pardinãs et al. 2005, Rocha et al. 2008). Didelphids are mostly arboreal, but may also forage on the ground, as documented for *Monodelphis glirina* (Brito and Fernandez 2002). In contrast, rodents have more terrestrial habits in dry tropical rainforests, mid-savanna and grassland habitats across their range (Adler 2000).

Amazonian small mammals have been resisting to forest fragmentation, surviving in metapopulations (Santos-Filho et al. 2012). However, the consequences for the different species regarding morphological variation and consequently morphological functionality under this fragmentation dynamic need to be clarified as data on how these processes influence individual condition is still relatively scarce for most vertebrates (Díaz et al. 1999; Mazerolle and Hobson 2002; Cattarino et al. 2016). Small mammals have been considered the ideal taxonomic group to answer questions at the landscape level due to the relative abundance of information on their biology, the ease of capture and marking, and the relatively "small" scale of the home range (Barret and Peles 1999). Because of this, the discussion on the perspectives to address questions on the landscape scale will be mainly based on small mammals. However, the ideas and methods can be applied to different taxonomic groups. In other words, these small mammal studies can help formulate landscape ecological principles.

In mammals, morphometric variation in individuals experiencing sub-optimal environmental conditions have been identified in skulls, mandibles and teeth, we analysed mandibles rather than the skull or teeth, in part because mandibular bones have been considered a model system more indicative of the natural history of mammals due to their masticatory function, and also because the mandible is composed of several morphogenetic units with different embryological origins and rates of differentiation (Harral 2003)

In this study, we aim to evaluate the impact of forest fragmentation on the mandible phenotype of four small mammal species (two species of Rodentia – *Proechimys longicaudatus* and *Necromys Lasiurus,* and two species of Didelphimorphia – *Marmosa demerarae* and *Monodelphis glirina*) inhabiting southern Amazonia. To this end we intend to evaluate mandibular morphometric variation in specimens inhabiting forest fragments of different sizes (small versus large) and continuous forest of this region. We here hypothesize that small mammals inhabiting smaller forest fragments show significant differences in mandible size and/or shape when compared with animals from larger fragments and continuous areas.

2.2.2. Material and Methods

2.2.2.1. Study area

The study area is in southern Amazonia, in Alta Floresta municipality, in the northernmost part of Mato Grosso state (Figure 1). The region was previously covered by rainforest and semi deciduous trees (Pires and Prance 1985) but is currently restricted to different size fragments surrounded by an open pasture matrix, encompassing around 40% of the state's area.

Sampling and data collection were performed in a total of 17 forest fragments and 2 continuous areas. Fragments were grouped according to size: ten small fragments (S) varying between 5 and 26 hectares, and seven large fragments (L), between 189 and 900 hectares. Two surrounding areas of continuous forest were chosen (C), both above 30,000 hectares (see details in Figure 1).

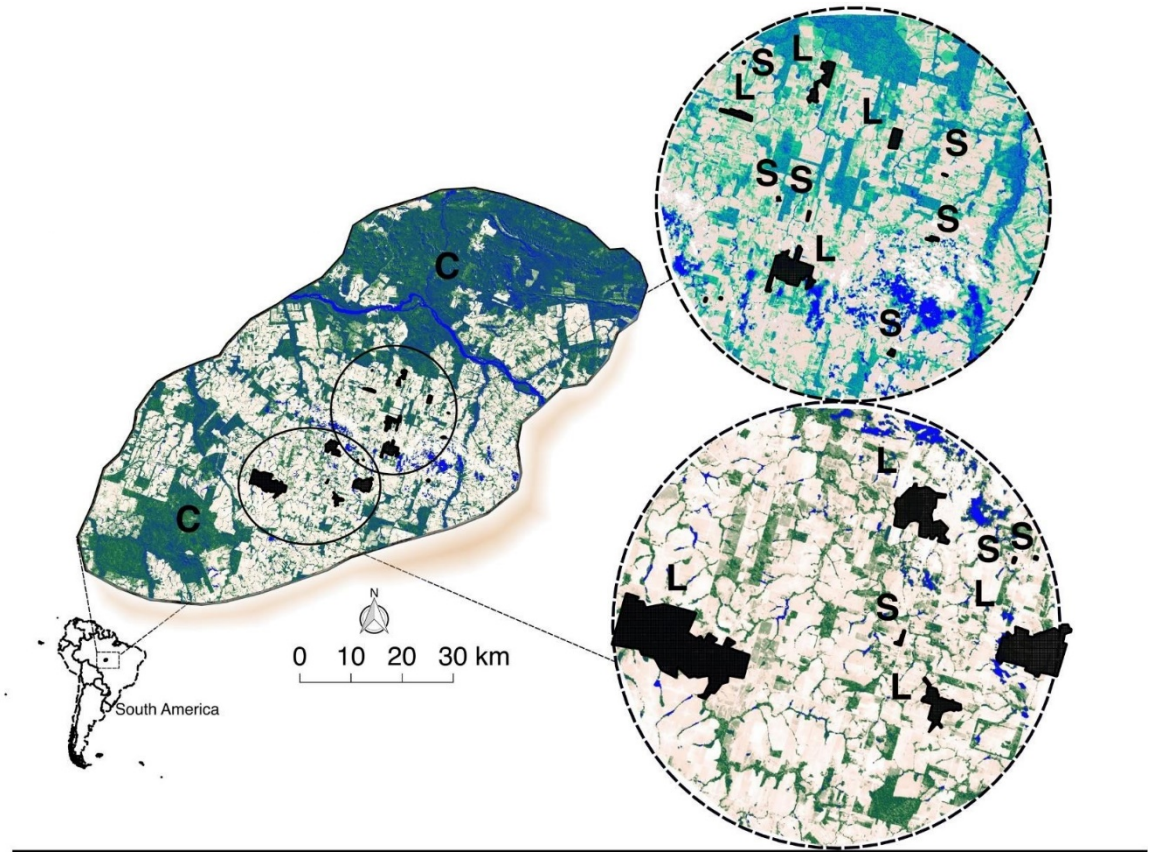


Figure 1. Study area in southern Amazonia exhibiting the spatial distribution of forest fragments (divided in small fragments – S, large fragments – L, and continuous areas – C).

2.2.2.2. Sample collection

A total of 334 adult individuals were sampled for morphometric analysis (see details in Table 1) across the study area: 143 specimens from the order Rodentia (81 long-tailed spiny rats, *Proechimys longicaudatus*, and 61 hairy-tailed bolo mice, *Necromys lasiurus*), and 191 specimens from the order Didelphimorphia (109 woolly mouse opossums, *Marmosa demerarae*, and 82 Amazonian red-sided opossums, *Monodelphis glirina*). Captures were carried out between May and September 2009, using Sherman traps (80 x 90 x 230 mm), Tomahawk traps (145 x 145 x 410 mm) and Pitfalls (60-litre buckets) (see sampling design in Castilheiro and Santos-Filho 2013). The animals were taxidermized and the mandibles cleansed by Dermestid beetles until ready to be photographed. The taxidermy and storage process were carried out at the Mammal Laboratory of the State University of the Mato Grosso (UNEMAT), Cáceres, Mato Grosso, Brazil, and compliance with the ethical rules for

the use of animals was duly followed with a license from the Brazilian governmental environmental agency IBAMA (collection license (nº 3998-1).

Table 1. Details of the sample analysed originating from a forest fragmented area in southern Amazonia

		Rodentia - Species (N)					
		Long-tailed spiny rat (*Proechimys longicaudatus*)			Hairy-tailed bolo mouse (*Necromys lasiurus*)		
Sites	Area (ha)	Females	Males	Total	Females	Males	Total
S	5 -26	20	18	38	10	20	30
L	189 - 900	18	14	32	11	20	31
C	>30,000	4	7	11	-	-	-
Total		42	39	81	21	40	61

		Didelphimorphia - Species(N)					
		Woolly mouse opossum (*Marmosa demerarae*)			Amazonian red-sided opossum (*Monodelphis glirina*)		
Sites	Area (ha)	Females	Males	Total	Females	Males	Total
S	5 - 26	45	22	67	24	26	50
L	189 - 900	13	11	24	11	21	32
C	>30,000	6	12	18	-	-	-
Total		64	45	109	35	47	82

Sites: S, Small fragments; L, Large fragments; C, Continuous forest.

2.2.2.3. Geometric morphometrics analyses

Both hemi-mandibles (right and left) were photographed in the mesial (lingual) view, with a digital camera, at a resolution of 20.1 megapixels, using the macro function without flash, always from the same height using a standard copystand (Muñoz-Muñoz et al. 2006; Muñoz-Muñoz et al. 2011; Franchini et al. 2016).

Twelve two-dimensional homologous landmarks were digitized on both left and right hemi-mandible of each specimen (see details in Figure 2, Table II) using TPSDig version 2 (Rohlf 2006). Landmarks were digitized three times in separate sessions by the same person and randomly ordering the specimens in order to minimize and quantify measurement error (Muñoz-Muñoz and Perpiñán 2010).

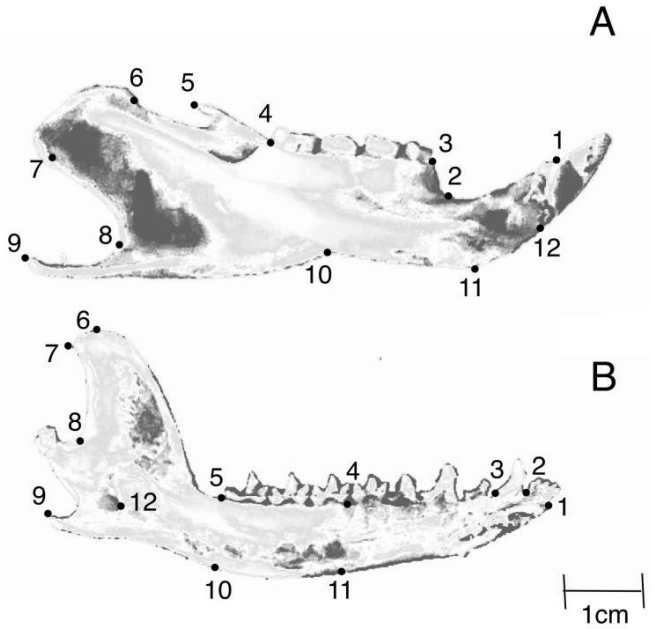


Figure 2. Twelve landmarks digitized on each hemi-mandible: A: Rodentia group represented by the long-tailed spiny rat (*Proechimys longicaudatus*). B: Didelphimorphia group represented by the woolly mouse opossum (*Marmosa demerarae*).

Coordinates of homologous landmarks were superimposed using a generalized least squares method (Generalised Procrustes Analysis, GPA), minimizing the sum of squares of distances between them (Dryden and Mardia 1998). This analysis removes from landmark coordinates the variation that is not due to shape, such as scale, position, and orientation (Rohlf and Slice 1990). We symmetrized landmarks on both sides of each hemi-mandible's mesial view and analysed the symmetric component of shape (Klingenberg et al. 2002).

The size of each hemi-mandible was estimated by the calculation of its Centroid Size (CS), the square root of the sum of squares of the distance between each landmark and the configuration's centroid (Zelditch et al. 2012). CS of each specimen was calculated as the mean value of the two sides and the three replicates.

Table 2. Location of landmarks in each hemi-mandible of the four small mammal species analysed.

Rodentia (A)	
Landmark	Location
1	Most cranio-dorsal point of the mandibular symphysis that meets the posterior part of the incisor's alveolar margin
2	Point of maximum concavity between the incisor's alveolus and the tooth row
3	Cranialmost point of the tooth row's alveolar margin
4	Caudalmost point of the tooth row's alveolar margin
5	Tip of the coronoid process
6	Cranialmost point of the edge of the condyle's articular surface
7	Caudalmost point of the edge of the condyle's articular surface
8	Point of maximum concavity between the condyloid and the angular process
9	Tip of the angular process
10	Point of maximum concavity of the mandible's ventral margin
11	Point of maximum convexity of the dentary in the cranio-ventral part
12	Most cranio-ventral point of the mandibular symphysis that meets the anterior part of the incisor's alveolar margin
Didelphimorphia (B)	
Landmark	Location
1	Base of the lower first incisor
2	Base of the lower fourth incisor
3	Posterior base of the lower canine
4	Posterior base of the first molar
5	Posterior base of the fourth molar
6	Central point in the coronoid process
7	Endpoint of the caudal border of coronoid process
8	Point of inflection of the curve between the mandibular condyle and the caudal border of the coronoid process
9	Highest point at end of side of the mandibular condyle
10	Landmark 5 orthogonal projection on the ventral edge of the mandible
11	Landmark 4 orthogonal projection on the ventral edge of the mandible
12	Foramen's edge

2.2.2.4. Statistical analysis

A variance analysis (Procrustes ANOVA) was carried out to evaluate the influence of measurement errors in shape and size. This method treats the independent variables - Individual (represents individual variation), Side (represents the Directional Asymmetry - DA) and Sex as random factors, and Procrustes distance as the dependent variable. The interaction between Side*Individual represents Fluctuating Asymmetry (FA). If this interaction (FA) encompasses significantly more variance than the residuals (residual variance across repetitions), the measurement error is considered negligible and a single set of landmarks digitization per mandible is necessary Klingenberg et al. 2002).

For testing mandible size differences, a variance analysis (ANOVA) of the logarithm of CS (log CS) between sexes and between forest fragment groups (Small, Large and Continuous) was conducted. For multiple comparisons of logCS, we used Tukey's test.

Allometry refers to the size-related changes of morphological traits, as concept, in geometric morphometrics, allometry is the covariation of shape with size (Klingenberg 2016). To explore the effect of size on mandibular shape we are using a size-shape analysis, we used the multivariate regression of shape variables on a measure of size (logarithm of CS), pooled by sex and or group (Small, Large, Continuous area).

Mahalanobis distance matrixes were constructed, with raw and size-corrected data among sexes (for Didelphimorphia) and between groups of areas (S, Small fragments; L, Large fragments; and C, Continuous areas) for all species (Rodentia and Didelphimorphia). For *P. longicaudatus* and *M. demerarae*) thre groups S, L, and C were formed. Due to the absence of specimens of *M. glirina* and *N. lasiurus* from Continuous areas, only two groups were considered (S and L).

In the case of two groups of areas (S and L), a Discriminant Function Analysis (DFA) was performed, including a parametric T-square test for the between-group means. We also used a permutation test (10 000 permutation runs), using the Mahalanobis distances (Rezić et al. 2017). For three groups of areas (S, L and C), a Canonical Variate Analysis (CVA) was

performed. Both the DFA and the CVA analyses were plotted with raw and size-corrected data (Rohlf et al. 1998, Klingenberg and Monteiro 2005).

All statistical analyses, including the geometric morphometric and generation of graphs, were carried out in MorphoJ version 1.06d (Klingenberg 2010) and R language (R Development Core Team 2018).

2.2.3. Results

2.2.3.1. Effects of fragmentation on mandible size and shape and size variation

2.2.3.1.1. Sources of size variation

Procrustes analyses of variance (ANOVAs) carried out on the replicated subsample for Didelphimorphia revealed a significant effect of the individual, sex and mandible side factors, as well as interaction individual*side, on mandible size (Table 3). For Rodentia species, a significant effect of the individual and the interaction individual*side was detected, but not the effect of side and sex on mandible size (Table 3). Since the measurement error was significantly lower that the interaction between side and individual (fluctuating asymmetry) the measurement error was considered negligible. Since sexual dimorphism was negligible for both *P. longicaudatus* and *N. lasiurus*, in each species data for both sexes were gathered for all subsequent analyses.

Table 3. Effect of sex, individual, mandible side, interaction between individual and side and measurement error on Log centroid size and shape of mandibles of rodents and didelphids inhabiting a forest fragmented area in Southern Amazonia. Results were obtained with a Procrustes ANOVA.

A – Rodentia						**B - Didelphimorphia**					
Long-tailed spiny rat (*Proechimys longicaudatus*)						**Woolly mouse opossum (*Marmosa demerarae*)**					
Log centroid size						**Log centroid size**					
Effect	SS	MS	df	F	P-value	Effect	SS	MS	df	F	P-value
Sex	2.48E-01	2.48E-01	1	0.13	0.724ns	Sex	1.52E+02	1.52E+02	1	42.06	0.0001**
Individual	1.57E+02	1.96E+00	80	47.1	0.0001**	Individual	3.87E+02	3.62E+00	107	78.00	0.0001**
Side	1.35E-02	1.35E-02	1	0.33	0.570ns	Side	3.02E+00	3.02E+00	1	65.06	0.0001**
Ind*Side	3.33E+00	4.16E-02	80	3.6	0.0001**	Ind*Side	5.01E+00	4.64E-02	108	2.88	0.0001**
Measurement error	3.74E+00	1.16E-02	324			Measurement error	3.70E-02	1.85E-02	2		
Shape						**Shape**					
Effect	SS	MS	df	F	P-value	Effect	SS	MS	df	F	P-value
Sex	3.01E-02	1.51E-03	20	2.8	0.067ns	Sex	1.16E-01	5.83E-03	20	14.85	0.0001**
Individual	8.81E-01	5.51E-04	1600	4.38	0.0001**	Individual	8.40E-01	3.92E-04	2140	14.41	0.0001**
Side	1.35E-01	6.75E-03	20	53.64	0.0001**	Side	1.17E-02	5.86E-04	20	21.51	0.0001**
Ind*Side	2.01E-01	1.26E-04	1600	8.1	0.0001**	Ind*Side	5.88E-02	2.72E-05	2160	6.79	0.0001**
Measurement error	1.01E-01	1.55E-05	6480			Measurement error	8.49E-05	2.12E-06	40		

Hairy-tailed bolo mouse (*Necromys lasiurus*)						**Amazonian red-sided opossum (*Monodelphis glirina*)**					
Log centroid size						**Log centroid size**					
Effect	SS	MS	df	F	P-value	Effect	SS	MS	df	F	P-value
Sex	1.87E-01	1.87E-01	1	0.36	0.552ns	Sex	3.31E+01	3.31E+01	1	8.19	0.0054**
Individual	3.15E+01	5.26E-01	60	32.6	0.0001**	Individual	3.23E+02	4.04E+00	80	143.65	0.0001**
Side	5.10E-02	5.16E-02	1	3.2	0.078ns	Side	3.32E-01	3.32E-01	1	11.79	0.0009**
Ind*Side	9.85E-01	1.61E-02	61	3.10	0.0001**	Ind*Side	2.28E+00	2.28E-02	81	2.46	0.0001**
Measurement error	1.28E+00	5.21E-03	247			Measurement error	3.75E+00	1.14E-02	328		
Shape						**Shape**					
Effect	SS	MS	df	F	P-value	Effect	SS	MS	df	F	P-value
Sex	4.32E-03	2.16E-04	20	0.35	0.996ns	Sex	6.60E-02	3.30E-03	20	3.60	0.0001**
Individual	7.31E-01	6.09E-04	1200	3.53	0.0001**	Individual	1.46E+00	9.18E-04	1600	15.83	0.0001**
Side	4.59E-02	2.29E-03	20	13.30	0.0001**	Side	2.03E-02	1.01E-03	20	17.53	0.0001**
Ind*Side	2.10E-01	1.72E-04	1220	8.40	0.0001**	Ind*Side	9.40E-02	5,80E-05	1620	10.32	0.0001**
Measurement error	1.01E-01	1.28E-05	4940			Measurement error	3.69E-02	5.62E-06	6560		

SS: sum of squares, df: degrees of freedom, MS: mean squares, F: F statistic, P-value – ns: not significant, **< 0.01; *< 0.05.

In both Rodentia species, no significant differences were found between sexes regarding the LCS (Table 4). As for Didelphimorphia, males in general were larger than females, although in large fragments the differences were not significant. For *M. demerarae* mandible was significantly larger in males than in females in small fragments and continuous areas, but not in large fragments differences in LCS were not significant. (see Figure3B, Table 4). In *M. glirina* this variable was also significantly higher in males than in females in small fragments but no significant differences between sexes in large fragments was found.

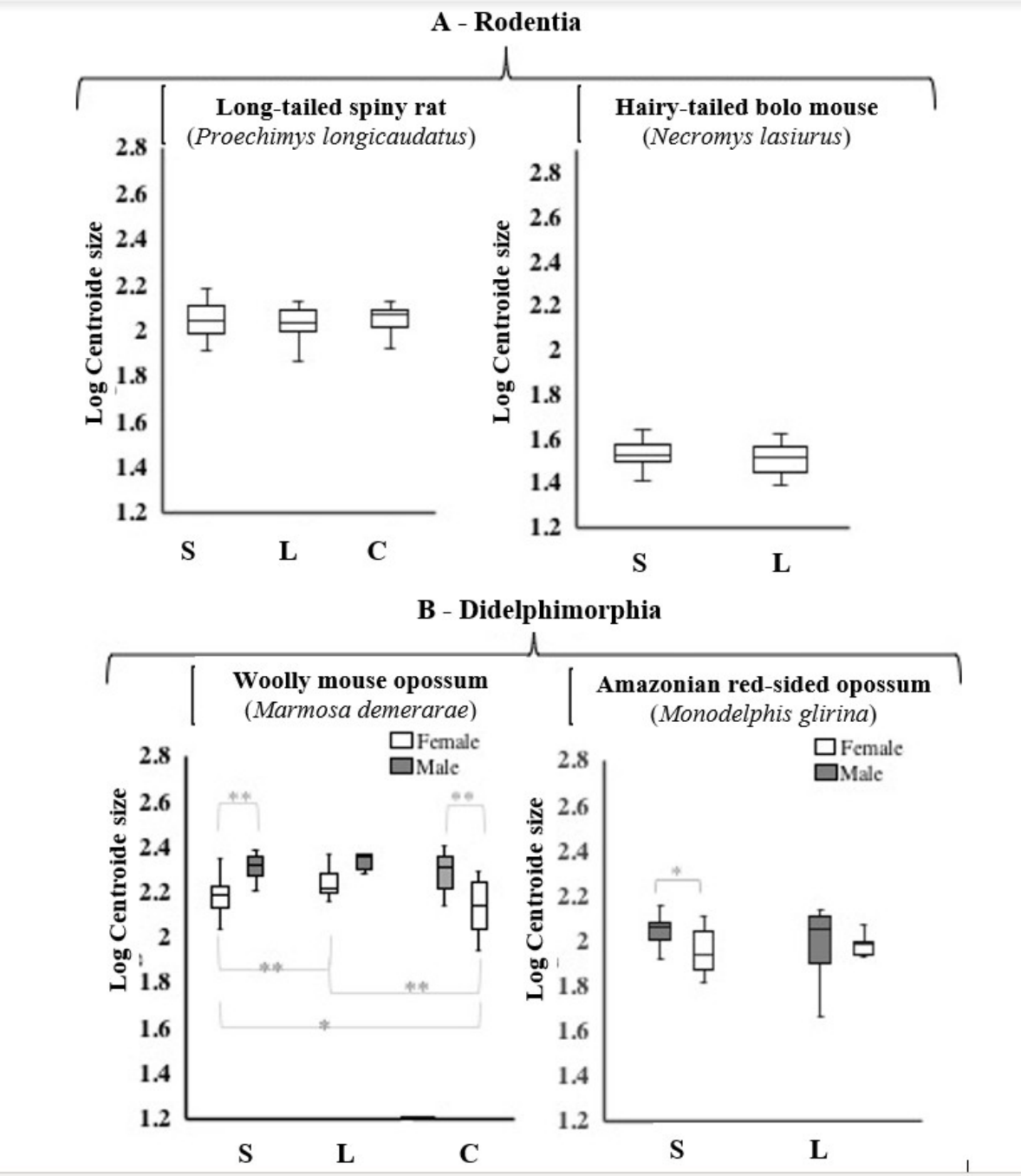


Figure 3. Mandible centroid size variability among fragments / continuous forest groups of A-Rodentia and B – Didelphimorphia. In each box, the horizontal line represents the median, box margins are at the 25th and 75th percentiles, bars extend to 5th and 95th percentiles, and the outlies were eliminated. The asterisks' number represent significance: ** P<0.001 and * P<0.005.

Table 4 ANOVA Results: Variation in centroid size between groups of fragments for both orders and between sex for the order Didelphimorphia.

	Didelphimorphia species, p-value			
	M. demerarae		*M. glirina*	
Groups	females	males	females	males
S-L	0.001**	0.29ns	0.92ns	0.81ns
S-C	0.043*	0.30ns	-	-
L-C	0.007**	0.99ns	-	-

	Rodentia species, p-value	
	P. longicaudatus	*N. lasiurus*
Groups		
S-L	0.44ns	0.43ns
S-C	0.99ns	-
L-C	0.59ns	-

	Didelphimorphia species, p-value	
	M. demerarae	*M. glirina*
Groups*Sex		
S.Female-S.Male	0.000**	0.038*
L.Female-L.Male	0.92ns	0.869ns
S.Female-L.Male	0.040*	0.316ns
L.Female-S.Male	0.047*	0.44ns
C.Male-C.Female	0.004**	-
C.Female-S.Male	0.000**	-
C.Female-L.Male	0.005**	-
L.Female-C.Male	0.39ns	-

S - Small fragments; L - Large fragments; C - Continuous forest; ns - not significant.

In both rodent species, no significant difference in LCS was observed in any pairwise comparison between fragment groups (*P. longicaudatus*: fragments S, L and C; *N. lasiurus*: fragments S and L) (see figure 3A, Table 4). As for Didelphimorphia, *M. demerarae* showed significant differences in LCS in all pairwise comparisons between groups, with females from large fragments showed significantly larger mandibles than those from the other fragment groups (small fragments and continuous areas. However, males of this species showed no significant difference in LCS in all pairwise comparisons. In the case of *M. glirina* no significant differences between fragment groups (small and large fragments) was found in LCS of both males and females (see Figure 3B, Table 4).

2.2.3.2. Allometry

The dependence of the shape's morphological variation on size (allometry) was significant between sexes with high explanation ‘percentages for both Didelphimorphia study species. The Size Effect (SE) on shape between males and females for *M. demerarae* was 41.14% (p-value=0.0001) and for *M. glirina* was 76.96% (p-value=0.0001) (see details on Table 5). Also, between fragments ‘groups, the dependence of the shape's morphological variation on size was significant for both Rodentia and Didelphimorphia orders. However, the percentages explaining the size effect (SE) on the shape were higher in Didelphimorphia species (SE in *M.demerarae* was for females 33%, p-value = 0.0001 and for males 54.17%, p-value 0.0001; in *M. glirina* for females was 76.31%, p-value = 0.0001 and for males 79.01, p-value = 0.0001) than Rodentia (SE for *P. longicaudatus* was 8.64, p-value = 0.0001 and for *N. lasiurus* 13.55, p-value = 0.0001 (see details in Table 5).

2.2.3.3. Sources of shape variation

Procrustes analyses of variance (ANOVAs) carried out on the replicated subsample for Didelphimorphia revealed a significant effect of the individual, sex and mandible side factors, as well as interaction individual*side, on mandible shape (Table 3). Since the measurement error was significantly lower that the interaction between side and individual (fluctuating asymmetry) the measurement error was considered negligible. For Rodentia species, a significant effect of the individual, side and the interaction individual*side were detected, but sex revealed a non-significant effect on mandible shape regarding shape only sex revealed a non-significant effect (Table 3). Since sexual dimorphism was negligible for both *P. longicaudatus* and *N. lasiurus*, in each species data for both sexes were gathered for all subsequent analyses.

Mandible shape was significantly different in both sexes of both species Didelphimorphia, considering both raw and size corrected data, shape morphological variation in the mandibles between sexes for both species are accentuated mainly in the ascending ramus, with the males having a greater amplitude in this structure in relation to the females (see Figure 4, Table 5). Contrarily, both rodent species did not exhibit sexual dimorphism regarding mandible shape variation (Table 3).

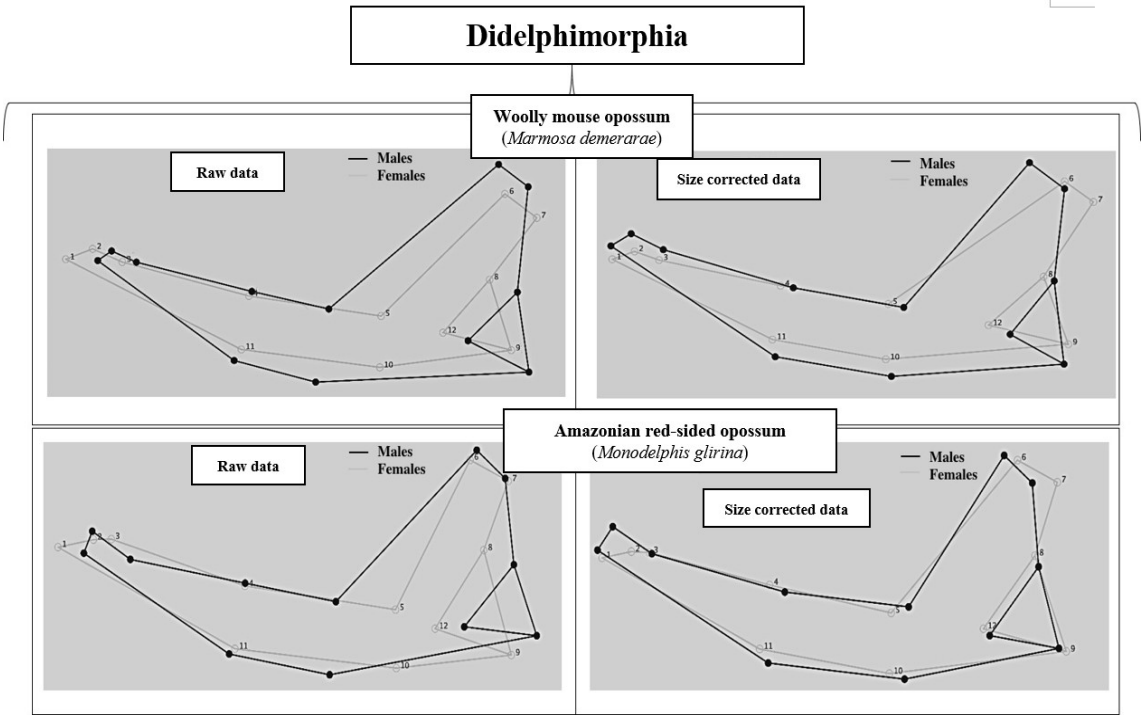


Figure 4. Mandible shape variation in male and female Didelphimorphia (*Marmosa demerarae* and *Monodelphis glirina*).

Mandible shape is significantly different (with both raw and size corrected data) regarding most pairwise comparisons between fragment size groups (S, L and C) for both rodents and didelphids. The only exception involved the long-tailed spiny rat (*Proechimys longicaudatus*), where no statistically significant differences were observed between animals from large fragments and continuous areas. For all other species, mandible shape variation was particularly impacted in small fragments, with significantly higher morphometric distances from large fragment and from continuous areas, when available for comparison. (see details in Figure 5, Table 5).

Table 5 Morphological variation in shape between sexes of the order Didelphimorphia and between groups of fragments for both orders.

Morphometric distances between sex for Didelphimorphia (Discriminant function between females and males)

	Sex	**Raw data**		**Size-effect (SE)**		**Size-corrected data**	
Didelphimorphia	**Groups**	**Mah.dist**	**P-value**	**Predicted (%)**	**P-value**	**Mah.dist**	**P-value**
Marmosa demerarae	F/M	2.05	0.0001**	41.14	0.0001**	1.68	0.0001**
Monodelphis glirina	F/M	2.03	0.0001**	76.96	0.0001**	1.94	0.0001**

Morphometric distances between groups of fragments (Canonical Variate Analysis between three groups (S, L, C) and Discriminant Function for two groups (S, L) results)

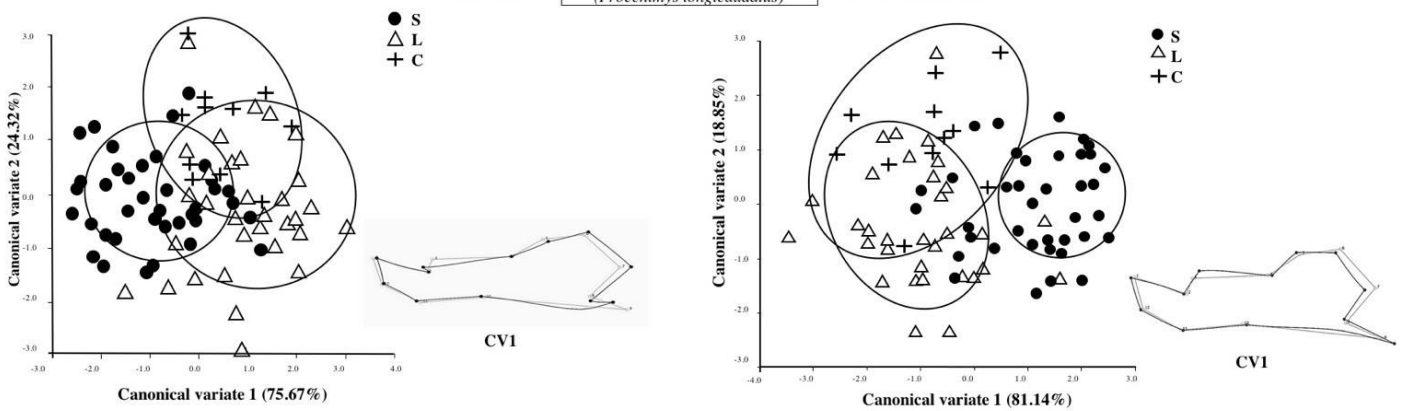
	Fragments	**Raw data**		**Size-effect (SE)**		**Size-corrected data**	
Rodentia	**Groups**	**Mah.dist**	**P-value**	**Predicted (%)**	**P-value**	**Mah.dist**	**P-value**
Proechimys longicaudatus	S-L	1.87	0.0001**	8.64	0.0001**	2.13	0.0001**
	S-C	1.90	0.04*			2.36	0.0001**
	L-C	1.62	0.30ns			1.58	0.370ns
Necromys lasiurus	S-L	2.3	0.0033**	13.55	0.0001**	2.28	0.0001**

Didelphimorphia	**Groups**	**Mah.dist**	**P-value**	**Predicted (%)**	**P-value**	**Mah.dist**	**P-value**
Marmosa demerarae – females	S-L	2.22	0.0001**	33	0.0001**	2.23	0.0001**
	S-C	3.75	0.0001**			3.72	0.0001**
	L-C	3.42	0.0019**			3.38	0.0029**
Marmosa demerarae – males	S-L	2.43	0.0001**	54.17	0.0001**	2.24	0.0006**
	S-C	2.30	0.0001**			2.17	0.0005**
	L-C	3.19	0.0019**			3.21	0.0001**
Monodelphis glirina – females	S-L	2.91	0.26ns	76.31	0.0001**	3.07	0.0001**
Monodelphis glirina – males	S-L	2.43	0.049*	79.01	0.0001**	2.37	0.0001**

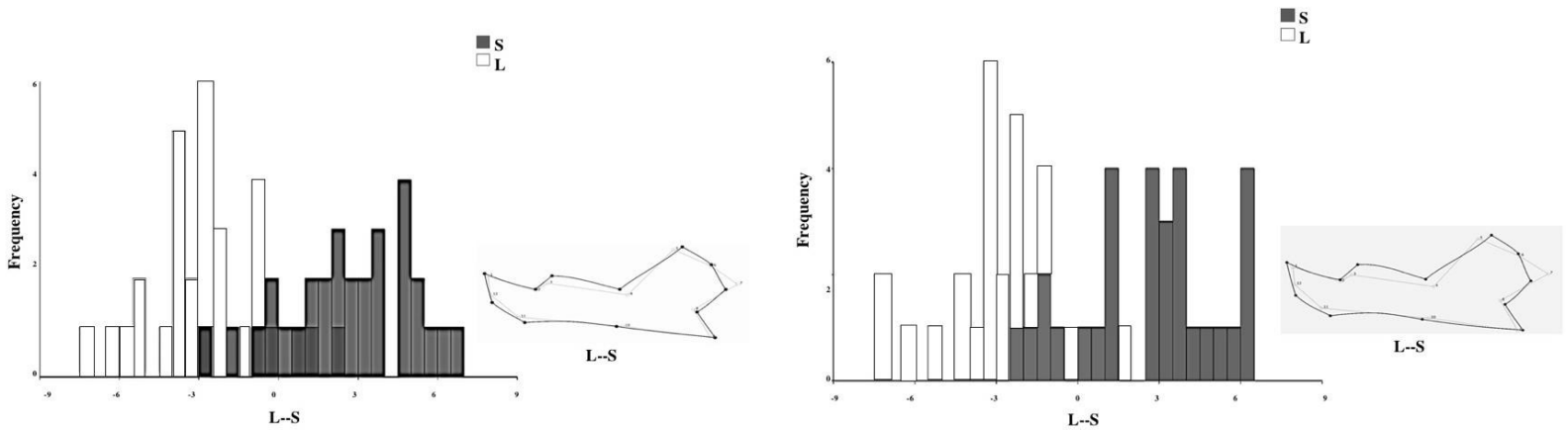
Obs: Mah.dist - Mahalanobis distance; F - females, M - males; S - small fragments, L - large fragments and C - Continuous area.

A- Rodentia

Long-tailed spiny rat
(Proechimys longicaudatus)



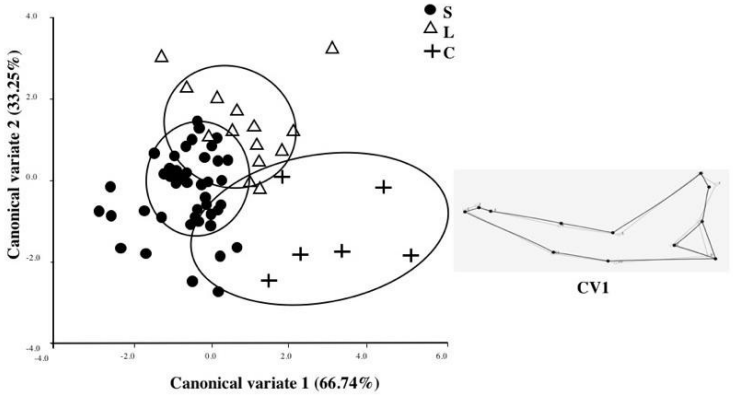
Hairy-tailed bolo mouse
(Necromys lasiurus)



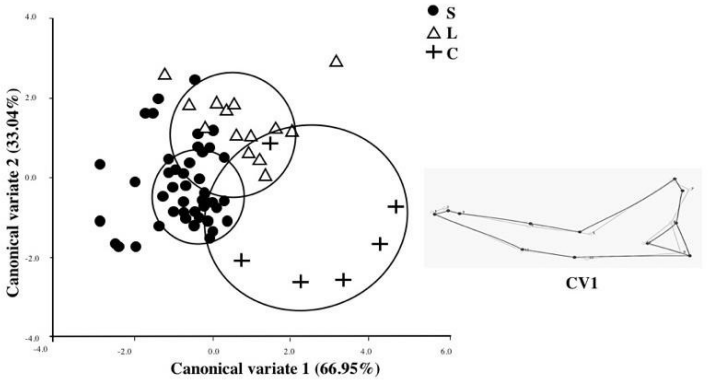
B- Didelphimorphia

Woolly mouse opossum
(Marmosa demerarae)

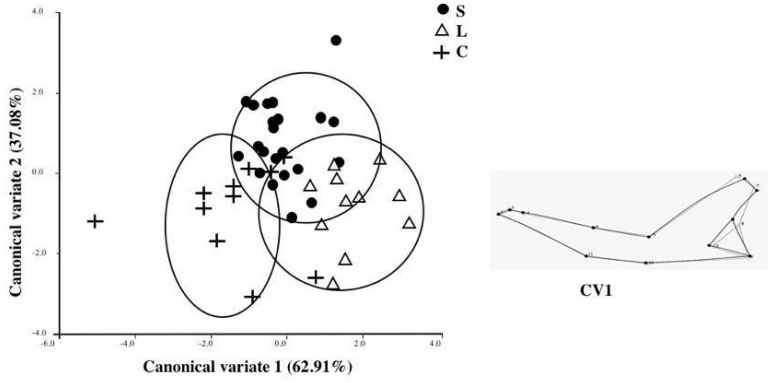
Raw data - Female



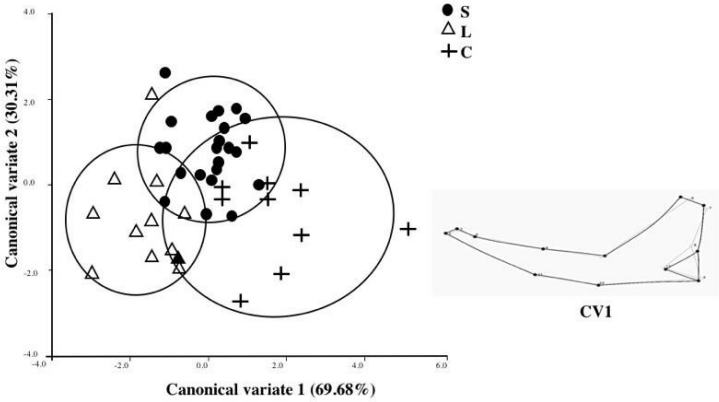
Size-corrected data - Female



Raw data - Male



Size-corrected data - Male



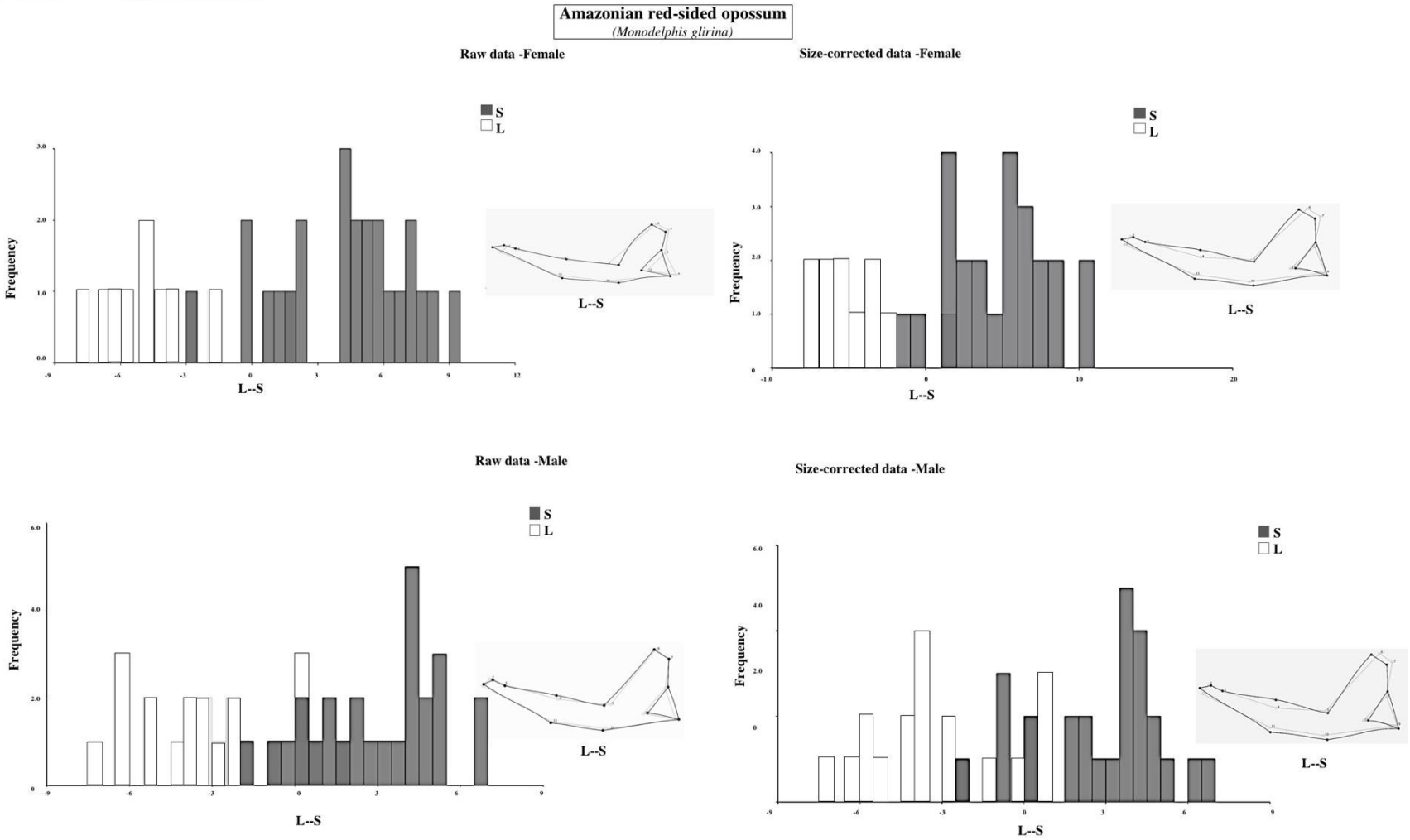


Figure 5. Mandible shape variation within and between groups of specimens inhabiting different size forest fragments in Southern Amazonia. Results were obtained from Discriminant Analysis (between two groups, S and L) and Canonical Variation Analysis (between three groups, S, L and C) for Rodentia and Didelphimorphia groups; S, Small fragments; L, Large fragments and C, Continuous area.

2.2.4. Discussion

We analysed size and shape mandible variation of two rodent and two didelphid species inhabiting a largely fragmented forest area in Southern Amazonia. Specifically, we compared this variation between specimens from populations inhabiting small and large forest patches and, whenever possible, continuous forest areas (data only available for *P. longicaudatus* and *M. demerarae*).

As expected, sexual dimorphism regarding mandible shape was not observed for both study rodents (*P. longicaudatus* and *N. lasiurus*). Concerning the mandible centroid size, in the rodent's species analysed, no significant differences were detected either between sexes or between different sized forest patches, i.e., fragmentation seems to have not significantly impacted mandible size. However, among our dataset, this was not always the case.

On the contrary, data from both didelphids (*M. demerarae* and *M. glirina*), revealed significant differences between sexes, with males being significantly larger than females, which is supported by a previous report based on skull and mandible analysis in *M. demerarae* (Astúa 2010) and the short-tailed opossum, genus *Monodelphis* (Pine et al. 1985; Chemisquy 2015). Results also indicate that this significant sexual dimorphism is also explained by allometry, i.e., the influence of size on shape (Table 5). Surprisingly, despite the already detected sexual dimorphism, only female *M demerarae* exhibited significant differences between fragment groups, being significantly smaller in continuous forest areas while males revealed no significant mandible size variation between any of the different sized fragments (see details in Figure 3, Table 4). The higher levels of migration movements reported for male woolly mouse opossums (Pires and Fernandez 1999) are probably resulting in a decreased differentiation in mandible size and shape between fragments (Figure 3, Table 4 and Figure 5, Table 5). As such, for this species, males may play an important role in decreasing the isolation effects of fragmentation.

Regarding shape, mandibles of mammals share certain developmental patterns, such as ontogenetic facial elongation, that are likely to constrain their shape variance (Caumul and Polly, 2005). Also, considering the mandible's functional role, no major morphological variation was expected in this structure. Nonetheless, it was clear that some of the observed

shape variation was explained by the differential size of forest patches from where animals originated. Shape wise, significant levels of differentiation were detected between groups, both in rodents and didelphids (Figure 5, Table 5). Based on this dataset, it was possible to verify that inhabiting small fragments has caused the most changes in individuals' mandible shape. Not only some level of separation was verified (Figure 5, Table 5), but it was also notable that for most study species, morphological variation within small fragments was the lowest, with a higher similarity observed among individuals (Figure 5, Table 5). This was particularly evident for *P. longicaudatus* and female *M. demerarae* as a third set of samples originating from continuous areas was also available for comparison. In these cases, morphometric distance to individuals from large fragments was significantly lower in comparison to those originating from small fragments.

Pardini (2004) considered that fragmentation can have some "positive effects" for some small mammals as some small fragments exhibit higher levels of abundance and species richness. This may be the case due to increased functional connectivity, habitat diversity, positive edge effects, stability of predator-prey systems, reduced competition and landscape complementarity (Fahrig 2017). Considering that most conservation zone selection algorithms are based on composition complementarity (Margules and Pressey 2000), small isolated fragments become prioritized because of their higher beta diversity. The consequences of these selections are extremely important for conservation purposes. However, large forest fragments are refuges for native fauna mainly in regions dominated almost exclusively by monoculture plantations (Chiarello 1999). Our results suggest that resident small mammal species in small fragments undergo significant morphological changes, namely a reduction in shape variance. To what extent these differences are also reflected in genetic impoverishment is still to be further analysed. However, specialist species as well as larger species, with broader home ranges or more dependent of the forest habitat, may struggle to survive in the long term, jeopardizing their persistence in the future. This highlights the need to evaluate this conservation selection method more carefully and considering data on a temporal scale. Obviously, each species has distinct ecological characteristics (Flores 2009, Nurtdinova and Pyastolova 2004), which complexify the decision of what to conserve. In fragmented habitats, varying patterns of species occurrences can be caused by their differential response to fragmentation (Lynam and Billick 1999),

increasing the need to search for patterns of populational response in this fragmented forest dynamics.

In Neotropical vertebrates, it has been proposed that nectarivores, possibly herbivores, and species able to use open habitats are affected significantly less by forest fragmentation than others (Vetter et al. 2011). As such, according to our results, the long-tailed spiny rat (*P. longicaudatus*) seems to be most resilient regarding the impacts of forest fragmentation on mandible shape, exhibiting the lowest morphometric distance between different sized fragments and the only one showing no significant differences between large fragments and continuous areas (see Table 5). Nevertheless, for this and all other species, the impact of small fragments was the most significant, with the highest recorded pairwise morphometric distances between groups mostly involving specimens originating from small fragments. On the other hand, the woolly mouse opossum (*M. demerarae*) revealed a less consistent pattern between different sized forest fragments in relation to differences in mandible size and shape variation. The morphometric distance between small fragments and continuous areas was lower than the distance between small and large fragments, which differ from the pattern observed in *P. longicaudatus*. For the remaining species, where no data existed for continuous areas, significant differences were always detected between small and large fragments, regardless of the fragments' relative position and distance between them. As already mentioned above, males of (*M. demerarae*) are more efficient at overcoming the matrix between fragments by successfully migrating between them (Pires and Fernandez 1999), which is in agreement with reports about their relative abundance in secondary forest and disturbed environments (Santos Filho et al. 2017). Curiously, among the study species, the woolly mouse opossum had the most arboreal habits (Brito and Fernandez 2002), but males seem to be able to use the trees in the middle of pasture and find paths that connect smaller with larger fragments or continuous areas. The other species probably mainly shelter at the fragment's edges (Santos-Filho et al. 2008), not being so successful at exploiting the pasture matrix.

In this study, based on empirical data, we reinforce the need for implementation of ecological corridors, not only with native trees but also underwood connecting fragments, allowing isolated populations to become metapopulations connected between fragments and source areas. This would maximize the chances for these species' persistence in the long term

by promoting gene flow between areas, reducing deleterious impacts of smaller forest patches, namely a substantial reduction in genetic diversity levels (Balkenhol et al. 2013, Lino et al. 2019). Here, we report phenotypic consequences in the form of morphologic alterations of the mandible, a critical structure for species survival. In this context, genome wide studies assessing the level of divergence between these fragmented small mammals' populations are essential to identify potential genes under selection and if those genes are somehow related with morphological development.

Results here presented were obtained from specimens collected in 2009 inhabiting a region subject to forest fragmentation since the early 1970's (Schmink and Wood 1997). Despite the small geographical scale of this study, it is clear that despite the short timeframe since the beginning of fragmentation, significant levels of morphological change have been detected. Factors such as local vegetation and diet can have a higher influence in mandible shape variation than mitochondrial DNA divergence (Caumul and Polly 2005). This highlights that genetic studies alone addressing divergence at neutral molecular markers may not necessarily pinpoint the deleterious effects of fragmentation.

In the absence of pre-fragmentation zoological collections, this dataset can constitute a valuable asset as a reference baseline for comparison in future studies as fragmentation proceeds and existing forest patches keep being reduced in size and continuous forest areas destroyed and fragmented. In the future, much can be learned by examining wildlife response in past episodes of environmental change. Possibly, the integration between spatial and temporal scales could provide important information on the differential response of different taxonomic groups, allowing the development of better informed and custom-made conservation measures. Small mammals and their assemblages are recognized as good indicators of ecological change and ecosystem's health (Rowe 2011), so these results may serve as a basis from a period closer to the beginning of the historical Amazon forest fragmentation.

2.2.5. Acknowledgments

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2.2.6. References

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3. Chapter 3

3.2. Fragmentation impacts on the fluctuating asymmetry of small mammals in southern Amazonia*

*Data in this chapter will be submitted as a paper to Symmetry

Fragmentation impacts on the fluctuating asymmetry of small mammals in southern Amazonia

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Abstract

In the southern Amazonia, habitat fragmentation constitutes one of the major causes of environmental stress for small mammals. Geometric morphometrics has proved to be a valuable tool to monitor mammal populations under distinct factors of environmental stress. In this context, the purpose of this paper is to examine the impacts of forest fragmentation on the patterns of FA (fluctuating asymmetry) (the intra and inter-population variability) in small mammal's mandibles (Rodentia and Didelphimorphia) present in southern Amazonia. This study was conducted in the municipality of Alta Floresta (Brazil), in the southern Amazonia. This area encompasses some of the most impacted and deforested regions of the Amazon forest, resulting in a hyper-fragmented landscape surrounded by pasture and some highly isolated patches. A total of 304 mandibles (left and right hemi-mandibles) originating from two species of Rodentia and two species of Didelphimorphia were used in this study. Analysis of FA was performed in digital images of the mandibles were withdrawn a view mesial after having been separated in the mandibular symphysis. Here, we tested whether the environmental variables: Fragment's size and isolation explain the variations in fluctuating asymmetry (FA) in four species of small neotropical mammals, two Rodentia: long-tailed spiny rat (*Proechimys longicaudatus*) and hairy-tailed bolo mouse (*Necromys lasiurus*) and two Didelphimorphia: woolly mouse opossum (*Marmosa demerarae*) and amazonian red-

sided opossum (*Monodelphis glirina*) inhabiting one of the most deforested and fragmented areas in the southern Amazonia. For the two largest species (long-tailed spiny rat (*Proechimys longicaudatus*) and woolly mouse opossum (*Marmosa demerarae*), among the studied, significant differences in FA were explained by the difference in fragment's size. High isolation' levels were positively correlated with high FA' levels, but non-significant in any of the cases. In this way, we suggest the use of FA as a biomonitoring tool for conservation biology, since it is a simple, inexpensive, in this case without sacrificing more animals and useful technique to measure stress and the influence of the fragmentation process on the phenotype of small mammal populations of the southern Amazonia.

3.2.1. Introduction

Habitat loss and fragmentation has long been recognized as a major driver for biodiversity depletion and ecosystem degradation (Haddad et al. 2015). In the southern Amazonia, habitat fragmentation constitutes one of the major causes of environmental stress for small mammals (Santos-Filho et al. 2016). The lack of connectivity between fragments can lead to a reduction in population size, an increase of inbred mattings and the interruption of gene flow (Seoane et al. 2000; Fahrig 2003).

The deleterious effects of forest fragmentation on species richness and abundance on Neotropical small mammal communities has been investigated in recent years (Rubio et al. 2014, Cerboncini et al. 2015; Gomez et al. 2015; Delciellos et al. 2015; Santos-Filho et al. 2016). Umetsu et al. (2008) suggest that matrix quality is important for the dynamics of fragmented landscapes and information about this can help understanding the community structure of small mammals and should be incorporated into modelling, allowing for more effective monitoring and management of tropical landscapes. Viveiros de Castro and Fernandez (2004) showed that tolerance to pasture vegetation separating fragments in Atlantic forest was the main determinant of the vulnerability to local extinction for small mammals, whereas other traits of the species were not good predictors of extinction vulnerability.

Santos-Filho et al. (2016) highlighted the importance of considering both taxonomic and habitat guild approaches using different taxa, with didelphids being positively associated

with vertical habitat structure, while rodents were more strongly related to a ground-level habitat structure. The presence of vegetation corridors connecting forest fragments has been addressed as an important factor for the persistence of movements of small mammals between fragments, resulting in similar species richness and abundance (Fialho et al. 2017).

Other authors reported the relevance of landscape metrics such as the fragment area and shape, edge size, and isolation in the species richness and abundance of small mammals (Michalski and Peres 2007; Vieira et al. 2009).

Some species of mammals may experience an increase in population density within smaller fragments. This "crowding effect" results of individuals surviving in the matrix migrating into persisting fragments (Debinski and Holt 2000). This phenomenon usually occurs soon after the fragmentation event but tends to disappear over time as a result of competition for the decreased resources. An impact in body size can also be observed as a consequence of habitat fragmentation, particularly in small forest fragments with restricted food availability or low habitat quality, resulting in smaller individuals (Debinski and Holt 2000).

Many studies addressing mammals in a context of forest fragmentation have focused on responses at the community level. However, very few surveys have evaluated whether these habitat changes and anthropogenic pressures cause morphological alterations on target species (Castilheiro et al. in prep; Manning and Chamberlain 1994; Wauters et al., 1996; Teixeira et al. 2006). These specimen-based approaches can help to detect changes in the performance of affected individuals and consequently in the structure of biological communities associated to the fragmented landscapes, potentially constituting sensitive bioindicators to be used in conservation and management of disturbed populations.

Geometric morphometrics has proved to be a valuable tool to monitor mammal populations under distinct factors of environmental stress (Teixeira et al. 2006; Lovatt and Hoelzel 2011; Askay et al. 2014; Coda et al. 2016), enabling the study of size and shape variation in morphological structures. Among these, fluctuating asymmetry (FA) results of embryonic developmental instability, during which subtle random deviations from symmetry occur, affecting bilateral structures (Leamy and Klingenberg 2005). Assessing FA as the

individual trait variation of left-right differences (e.g. mandibles, wings), allows the evaluation of disturbance levels experienced during the development of characters affecting the ability of an organism to develop uniform traits that are presumably controlled by an identical set of genetic instructions (Palmer and Strobeck 1986; Klingenberg 2015).

Severe environmental stress seems to be necessary to induce significant levels of FA, but the detection of environmental effects in wild populations is not straightforward (Graham et al. 2010). Many environmental changes, either as a result of anthropogenic pressures or other kind, can induce FA in mammals. For example, Sánchez-Chardi et al. (2013) concluded that shrews (*Crocidura russula*, Hermann 1780) from polluted areas show higher levels of asymmetry than those in less disturbed natural ecosystem. Oleksyk et al. (2004) reported that wood mice (*Apodemus flavicollis*) inhabiting the vicinities of the Chernobyl failed nuclear reactor show greater levels of asymmetry associated with radioactive contamination. Many others, including not so conspicuous disturbances can also increase FA levels, such as the temperature changes, audiogenic stress and protein deprivation increased FA in the first molars of Wistar rats (Sciulli et al. 1979). Mateos et al. (2008) showed an example where FA is negatively correlated to the individual condition (size and body mass) of Iberian red deer (*Cervus elaphus hispanicus*).

Genetic stress can also result in a FA increase, as high levels of homozygosity are also associated with increased FA and there is speculation whether inbred mattings also contribute to the reduction of developmental stability (Merola 1994; Vollestad et al. 1999; Özener 2010).

When in synergy, genetic and environmental stress promoted by forest fragmentation can cause a great increase in FA, especially when populations are totally isolated. In this context, FA seems to be a good biological indicator of fragmentation-imposed stress (Parsons 1992, Beasley et al. 2013; Helle et al 2013).

The fragmentation process invariably results in changes in the physical environment such as the edge effect or a sometimes-dramatic reduction of suitable habitat area, with consequences at the populational level but also with deleterious effects at the individual level, affecting natural levels of FA in animals. Lens et al. (1999) demonstrated in afrotropical birds

that FA increased in more degraded habitats under a forest fragmentation scenario. Anciães and Marini (2000) reported an increase in wing and tarsus FA in birds inhabiting forest fragments when compared with continuous areas and that in both structures, FA was negatively correlated with fragment size. Teixeira et al. (2006) also showed that most impacted environments resulted in significantly higher levels of FA using a new method of quantitative evaluation of temporal effects of anthropic impacts on didelphids.

In this context, the goal of this study is to examine the pattern of intra and inter-population FA levels in mandibles of four small mammals (Rodentia and Didelphimorphia) inhabiting a highly fragmented area in southern Amazonia. We also intend to assess the potential of using FA as a biomonitoring tool in conservation biology applicable to natural populations of small mammals inhabiting fragmented habitats.

3.2.2. Material and Methods

3.2.2.1. Study area

This study was conducted in the municipality of Alta Floresta (Brazil), in the southern Amazonia (see Figure 1). This area encompasses some of the most impacted and deforested regions of the Amazon forest, resulting in a hyper-fragmented landscape surrounded by pasture and some highly isolated patches.

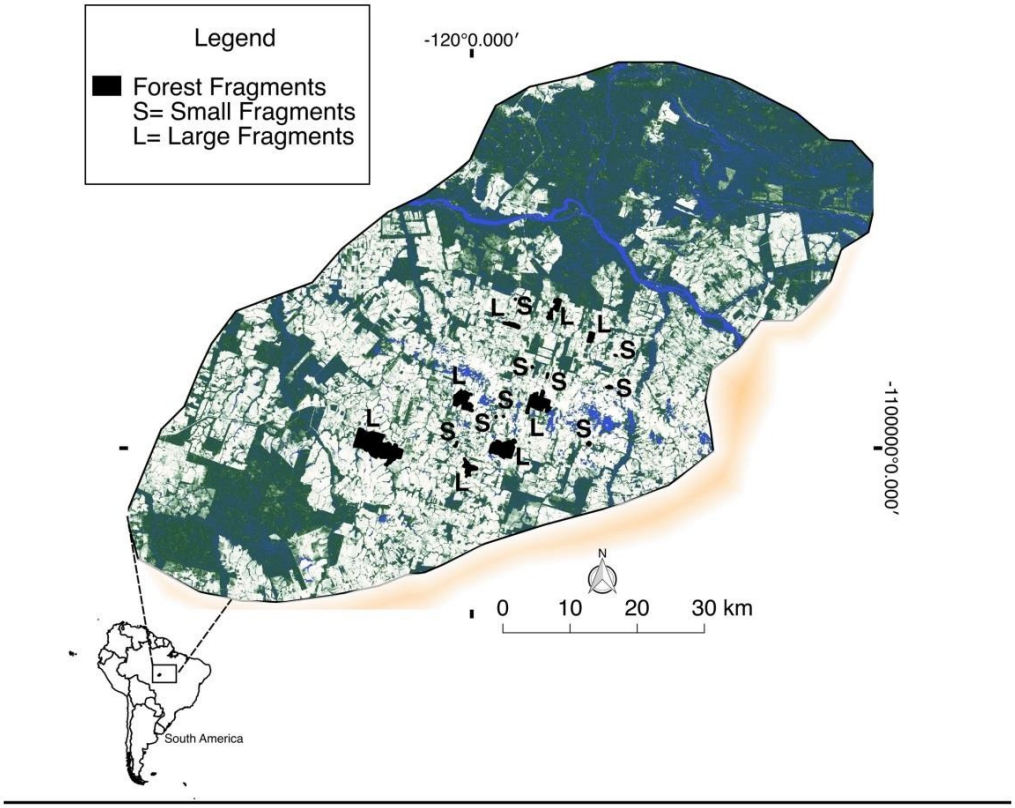

Figure 1. Study area in southern Amazonia.

In the region, the predominant vegetation is Ombrophylous open forest type and currently the main economic activity is livestock. The study area is located is the sub-basin of Teles Pires river, tributary of the Tapajós river, identified as a priority area for conservation and recovery in Agenda 21 (United Nations Conference on Environment and Development).

3.2.2.2. Sampling and data collection

A total of 304 mandibles (left and right hemi-mandibles) originating from two species of Rodentia and two species of Didelphimorphia were analysed in this study. The total dataset comprised 131 specimens of Rodentia: 70 mandibles of the long-tailed spiny rat (*Proechimys longicaudatus*) and 61 mandibles of hairy-tailed bolo mouse (*Necromys lasiurus*); and 173 specimens of Didelphimorphia: 91 mandibles of woolly mouse opossum (*Marmosa demerarae*), 82 mandibles of amazonian red-sided opossum (*Monodelphis glirina*) (see details in Table 1).

Table 1 Sampling details of small mammals' pairs of hemimandibles originating from a forest fragmented area in southern Amazonia.

		Rodentia - Mandibles (N)					
		Long-tailed spiny rat (*Proechimys longicaudatus*)			Hairy-tailed bolo mouse (*Necromys lasiurus*)		
Sites	Area (ha)	Female	Male	Total	Female	Male	Total
S	5 - 26	20	18	38	10	20	30
L	189 - 900	18	14	32	11	20	31
Total		38	32	70	21	40	61

		Didelphimorphia - Mandibles (N)					
		Woolly mouse opossum (*Marmosa demerarae*)			Amazonian red-sided opossum (*Monodelphis glirina*)		
Sites	Area (ha)	Female	Male	Total	Female	Male	Total
S	5 - 26	45	22	67	24	26	50
L	189 - 900	13	11	24	11	21	32
Total		58	33	91	35	47	82

Obs: Sites, S - Small fragments, L - Large fragments

All analysed specimens were adults and originated from a collection held at the Mammalogy lab at the State University of Mato Grosso (UNEMAT, Brazil). Specimens were collected between May and September 2009, from 17 forest fragments ranging from 5 to 900 hectares (h). Spatial distance between-fragments varied between 4 and 51 km.

Fragments were grouped by a size range (Small: 5-26 hectares and Large: 189-900 hectares). The study area is embedded in the forefront of the so-called 'arc of deforestation' in the Brazilian southern Amazonia, located in the municipality of Alta Floresta, Mato Grosso (see Figure 1).

3.2.2.3. Geometric Morphometrics: estimates of fluctuating asymmetry

The core idea about FA as a measure of developmental instability (DI) is that both sides of an organism can be viewed as independent replicas of the same developmental event. Both

sides share the same genotype and in a homogeneous environment (i.e. identical on both sides), they are under the influence of the same external factors. During development, small random perturbations cause the developmental pathway to deviate from its expected trajectory under the given environmental conditions. As these processes act locally, thereby affecting only one body part, their effects will accumulate on left and right side separately, leading to asymmetric phenotypes. The sensitivity to random perturbations can be viewed as the tendency of a developmental system to produce a morphological change in response to these perturbations and is often called developmental instability (e.g. Klingenberg 2003, Nijhout and Davidowitz 2003). Developmental instability is expressed phenotypically by within-individual variation, which is traditionally measured by FA in bilaterally symmetric organisms, as is the case in this study.

Analysis of FA was performed in digital images of the mesial view of mandibles separated through the mandibular symphysis into left and right hemi-mandibles, previously cleaned by Dermestid beetles. High resolution photographs (20.1 megapixels) were taken, always under the same conditions, at the same height aided by a copy-stand and using a Sony A5000 camera, (Muñoz-Muñoz et al. 2006; Muñoz-Muñoz et al. 2011; Franchini et al. 2016).

Twelve landmarks were placed on the images of each hemi-mandible on the lingual side encompassing its shape (see Figure 2 and Table 2 for the description of each landmark's location) using the tpsDig.2 software (Rohlf 2006). Three replicates were used for each set of hemi-mandibles.

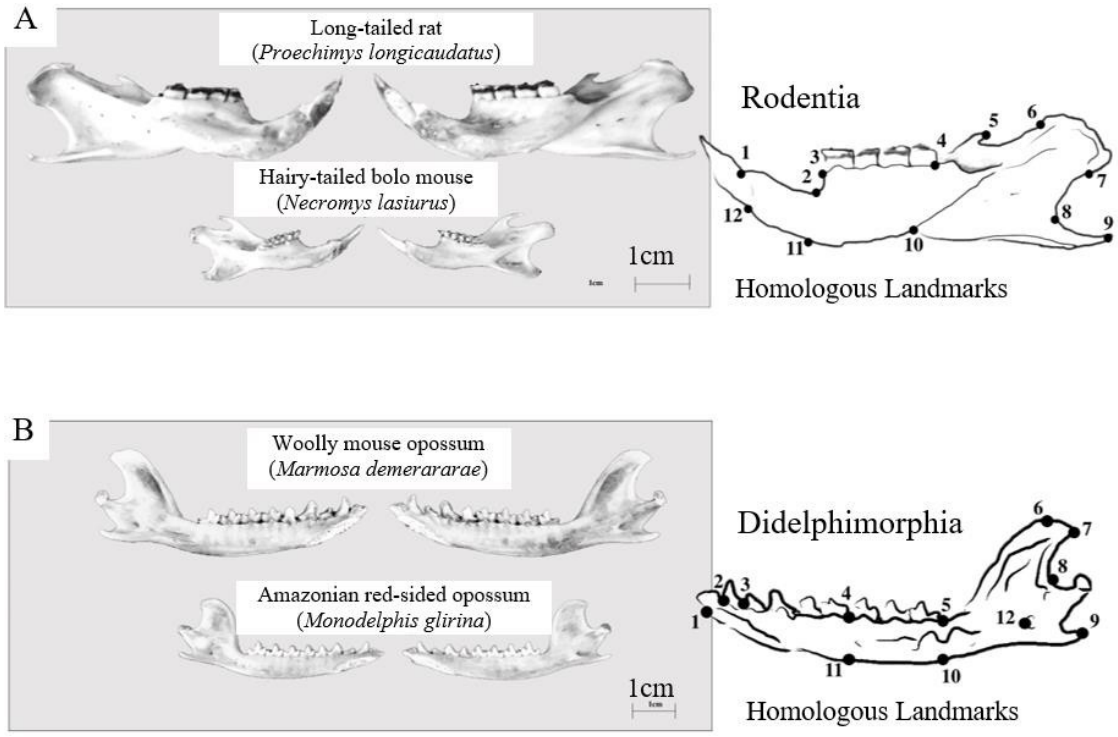

Figure 2. Relative differences between Rodentia (A) and Didelphimorphia (B) species in terms of mandible size and shape; representation of the lingual view of the right mandible of a i) rodent and a ii) didelphid showing the location of the selected landmarks for rodents (12 landmarks).

Table 2 Location of landmarks in each hemi-mandible of the four small mammal species analysed.

Rodentia (A)	
Landmark	Location
1	Most cranio-dorsal point of the mandibular symphysis that meets the posterior part of the incisor's alveolar margin
2	Point of maximum concavity between the incisor's alveolus and the tooth row
3	Cranialmost point of the tooth row's alveolar margin
4	Caudalmost point of the tooth row's alveolar margin
5	Tip of the coronoid process
6	Cranialmost point of the edge of the condyle's articular surface
7	Caudalmost point of the edge of the condyle's articular surface
8	Point of maximum concavity between the condyloid and the angular process
9	Tip of the angular process
10	Point of maximum concavity of the mandible's ventral margin
11	Point of maximum convexity of the dentary in the cranio-ventral part
12	Most cranio-ventral point of the mandibular symphysis that meets the anterior part of the incisor's alveolar margin
Didelphimorphia (B)	
Landmark	Location
1	Base of the lower first incisor
2	Base of the lower fourth incisor
3	Posterior base of the lower canine
4	Posterior base of the first molar
5	Posterior base of the fourth molar
6	Central point in the coronoid process
7	Endpoint of the caudal border of coronoid process
8	Point of inflection of the curve between the mandibular condyle and the caudal border of the coronoid process
9	Highest point at end of side of the mandibular condyle
10	Landmark 5 orthogonal projection on the ventral edge of the mandible
11	Landmark 4 orthogonal projection on the ventral edge of the mandible
12	Foramen's edge

Values of FA were calculated based on the Procrustes coordinates (x, y) representing the differences between coordinates of the best fit landmark configuration and the corresponding superimposed landmarks after the Procrustes analysis. Superimposition involved the reduction of homologous landmarks on the left and right hemimandible to a single configuration by mirror-imaging one of the hemimandible and then using the least-squares method to minimize differences in the positions of all landmarks among all configurations (removing the effects of size, translation and rotation) (Klingenberg and McIntyre 1998).

To calculate the Landscapes metrics at the level of the fragments we use the plugin Landscape Ecology Statistics (LecoS) in Qgis 2.01 (Jung 2016). We use a combination of two files in a raster, polygons (vector point-fragments) plus a Landsat previously classified provided by U.S. Geological Survey. Calculated metrics with the plugin were the Area, Edge length (Total length of edge or perimeter), and Shape index (or Fractal dimension index: a fractal dimension greater than 1 for a 2-patch, indicates an increase in shape complexity. Shape index in this case, approaches 1 for shapes with very simple perimeters such as squares and approaches 2 for shapes with highly convoluted, plane-filling perimeters), a detailed description of the calculated metrics can be found at McGarigal et al. (2012).

3.2.2.4. Estimates of Isolation index

The Isolation index, as proposed by Metzger (1998), was calculated considering the size (area) and proximity of all neighbouring fragments whose edges are within a specified radius of the focal fragment.

$$\text{Isolation index} = \left(\frac{(d1)2}{(A0) * (A1)}\right) + \left(\frac{(d2)2}{(A0) * (A2)}\right) + \left(\frac{(d3)2}{(A0) * (A3)}\right) + \left(\frac{(dx)2}{(A0) * (Ax)}\right)$$

In this formula "A0" refers to the area of each of the chosen fragments, "A1, A2, A3, Ax" to the area of neighbouring fragments, and "d1, d2, d3, dx" to the distances of the neighbouring fragments. We considered two scenarios for the index calculation concerning the length of the chosen radius surrounding each fragment: neighbouring fragments with area >2 hectares within a 2 km or 5 km radius from the centre of each sampled fragment.

Isolation index was calculated for each sampled fragment considering 2km and 5 km of distance to surrounding forest fragments As results for the furthest distance (5 km, data not shown) were very similar to those obtained for the 2 km, subsequent analysis only considered the 2 km radius.

A review of existing species ecology information was performed, data on (Habitat, Diet, Reproduction, Home range and Distribution (see Table 3)).

Table 3 Traits of both study rodents and didelphids.

Order	**Rodentia**	
Species	***Proechimys longicaudatus***	***Necromys lasiurus***
Habitat	Ground-dwelling (Machado et al. 2005)	Ground-dwelling (Pires et al. 2010)
Diet	Omnivorous/Seeds (Carvajal and Adler 2008)	Granivore/Seeds (Francisco et al. 1995; Magnusson et al. 1995)
Reproduction	All year	Reproductive activity increases during the rainy seasons (January to March) compared to the dry seasons (July to September). Reproductive rate probably follows rainfall, with an average of 3-6 young per litter (Francisco et al. 1995; Cangussu et al. 2002)
Home range (h)	0.11-0.15 h (Adler 2000)	Males home range: 0.41 - 2.1 h. Females home ranges being approximately 35% the area of male home ranges (Magnusson et al. 1995)
Average naso-anal length (cm)	20.0 cm	10.70 cm
Distribution	South of Bolivia, the North Paraguay and Central Brazil	Central Brazil south of the Amazon River, extreme southeast Peru, and northeast Argentina. It also occurs in Paraguay and Bolivia.
Order	**Didelphimorphia**	
Species	***Marmosa demerarae***	***Monondelphis glirina***
Habitat	Arboreal (Brito and Fernandez 2002)	Ground-dwelling (Nowak 1999)
Diet	Omnivorous (Fernandes et al. 2006)	Omnivorous/Insects (Castilheiro and Santos-Filho 2013)
Reproduction	Only during the rainy season (Nowak 1999)	Up to 4 litters per year (7 young per litter); breeding season typically from May to August (Nowak 1999)
Home range (h)	Females: ~0.10 h; males: can overlap the	0.12 -0.18 h

	range of different populations, being less territorial than females (Pires and Fernandez 1999; Quental et al. 2001)	
Average naso-anal length (cm)	16.98 cm	12.54 cm
Distribution	From Peru, Bolivia, Colombia, Venezuela through Guyana, to northern and central Brazil	Northeastern Brazil in the state of Pará, south of the Amazon river, through southern Perú and northern Bolivia

3.2.2.5. Statistical analyses

When analysing FA, it is important to perform a preliminary analysis to discard other types of asymmetry (Palmer and Strobeck 1986), such as directional asymmetry (DA) and antisymmetry (AS) (for details see e.g. Klingenberg 2015). To check if within-individual variation (FA and DA) was significant, parametric F-tests were applied in Procrustes and two-factor ANOVAs.

We performed a variance analysis (Procrustes ANOVA) using three replicated landmark sets to evaluate the influence of measurement error. This considers the independent variables 'Individual' (representing individual variation), 'Side' (representing directional asymmetry - DA) as random factors and Procrustes distance as the dependent variable. The interaction between Side*Individual represents fluctuating asymmetry (FA), quantifying the error from the residual variance across repetitions (Muñoz-Muñoz and Perpiñán 2010).

To check for antisymmetry, the logarithm of centroid size asymmetry (log CS asymmetry) and asymmetric components of shape variation were investigated for signs of deviations from normal distribution using the Shapiro-Wilk test and calculating the values of kurtosis (kurt 3= mesokurtic distribution, kurt >3 = platykurtic distribution and kurt <3 = leptokurtic distribution) according to Anscombe and Glynn (1983). All these analyses were conducted separately for each specie.

Allometry is the dependence of shape on size and can potentially affect the results on shape differences. Allometry was assessed through multivariate regression using the asymmetric components of shape onto the logarithm of the asymmetric centroid size (log CS asymmetry). Statistical significance of regression was obtained through permutation tests with 10,000 iterations under the null hypothesis of independence between size and shape (Klingenberg, 2011).

Statistical comparisons of FA were accomplished through analysis of variance (ANOVA) with planned contrasts, Kruskal-Wallis tests, with focus in scientifically sensible comparisons rather than every possible comparison, making only a limited number of comparisons, we increase the statistical power of each comparison. Since no significant differences were found between sexes regarding FA, data from both males and females was pooled for subsequent analyses. Variation in the FA levels between fragment ‘groups (Small and Large fragments) were tested for each specie.

PCA (Principal Component Analysis) type used during the computations was the Pearson's correlation matrix, used to correlate the average FA of each species with the landscape′s metrics (Area, Edge length, Shape index and Isolation index) calculated. The correlation coefficient (**CC**) ranges from -1 to 1. A value of 1 implies that a linear equation describes the relationship between Landscape metrics (x) and the FA (y) perfectly, with all data points lying on a line for which Y increases as X increases. A value of −1 implies that all data points lie on a line for which Y decreases as X increases. A value of 0 implies that there is no linear correlation between the variables.

The original variables that have the most significant factor loadings in each factor are the ones that contribute the most to the nomination, a factorial load is said to be significant when its value, in module, is higher than 0.60. We choose to activate the option to display significant correlations in bold characters (significance level 5%) (Pearson 1901).

Linear regressions were made for estimating the relationship between mean FA in each fragment and Isolation Index.

All statistical analyses were carried out using MorphoJ version 1.06d (Klingenberg 2011), R language (R Development Core Team 2018) and the XLSTAT 10:12 tool.

3.2.3. Results

Regarding Rodentia, in small and large fragments, the distribution of logCS asymmetry was non-normal for the long-tailed spiny rat (*Proechimys longicaudatus*): (Small fragments, W= 0.916, p-value= 0.007; Large fragments, W= 0.911, p < 0.012) and platykurtic (Small fragments: Kurt=3.04; Large fragments: Kurt= 3.07). Likewise, for the hairy-tailed bolo mouse (*Necromys lasiurus*): (Small fragments: W= 0.927, p-value= 0.042; Large fragments: W=0.948, p-value= 0.139ns), although the normality test was not significant in Large fragments, for both groups the distribution was leptokurtic (Small fragments: Kurt= 1.98; Large fragments: Kurt= 2.49).

Regarding Didelphimorphia, in small fragments, the distribution of logCS asymmetry was non-normal and in large fragments was normal for the woolly mouse opossum (*Marmosa demerarae*): (Small fragments: W= 0.940, p-value= 0.003; Large fragments: W= 0.953, p-value= 0.294ns), being platykurtic (Small fragments: kurt= 3.57), and although the normality test was not significant in Large fragments, the distribution was leptokurtic (Large fragments: kurt= 2.959). In Small and Large fragments, the distribution of logCS asymmetry was non-normal for specie amazonian red-sided opossum (*Monodelphis glirina*) (Small fragments: W= 0.881, p-value= 0.0001, Large fragments: W= 0.899, p-value = 0.006), platykurtic and leptokurtic, respectively (Small fragments: kurt= 4.54; Large fragments: kurt= 2.0012).

In small and large fragments, for both species of Rodentia and Didelphimorphia, all the 96 distributions resulting from the asymmetric component of shape variation revealed a normal distribution (p-value≥0.05) and mesokurtic (Kurt= 3).

These results reveal antisymmetry concerning the mandible's size but not the shape of all study species. In this way, considering that the shape distributions are of the mesocurtic type, i.e., have the same flattening as the normal distribution, data indicates that asymmetry in shape is of the fluctuating type. Therefore, further FA analyses were performed only concerning shape.

Considering the asymmetric component, a non-significant dependence of shape on size (allometry) was detected regarding both for sex and site/fragment (Table 4).

Table 4 Results from Allometry that was assessed through multivariate regression using the asymmetric components of shape onto the logarithm of the asymmetric centroid size (log CS asymmetry).

Species	Pooled by sex		Pooled by group	
	Size Effect		Size Effect	
Rodentia	Predicted (%)	*P-value*	Predicted (%)	*P-value*
Proechimys longicaudatus	2.33	0.17ns	2.33	0.17ns
Necromys lasiurus	0.41	0.61ns	0.66	0.51ns
Didelphimorphia	Predicted (%)	*P-value*	Predicted (%)	*P-value*
Marmosa demerarae	0.20	0.64ns	0.06	0.79ns
Monodelphis glirina	0.00	0.99ns	0.04	0.84ns

Since this interaction (FA) has significantly more variance than the residual, the error can be considered negligible and a single digitization of landmarks by mandible can be used (see Table 5).

Table 5 Procrustes ANOVA results: conducted on the replicates to evaluate the influence of measurement error on shape and centroid size data.

A - Rodentia						**B - Didelphimorphia**					
long-tailed spiny rat (*Proechimys longicaudatus*)						woolly mouse opossum (*Marmosa demerarae*)					
Centroid size						**Centroid size**					
Effect	SS	MS	df	F	P	Effect	SS	MS	df	F	P
Individual	1.45 E+02	2.07 E+00	70	50.22	0.0001**	Individual	4.16 E+02	4.63 E+00	90	78.00	0.0001**
Side	1.70 E-02	1.70 E-02	1	0.41	0.523ns	Side	2.37 E+00	2.37 E+00	1	65.06	0.0001**
Ind*Side	2.89 E+00	4.13 E-02	70	3.54	0.0001**	Ind*Side	4.19 E+00	4.65 E-02	90	2.88	0.0001**
Measurement error	3.31 E+00	1.16 E-02	284			Measurement error	3.70 E-02	1.85 E-02	2		
Shape						**Shape**					
Effect	SS	MS	df	F	P	Effect	SS	MS	df	F	P
Individual	7.64 E-01	5.45 E-04	14000	4.63	0.0001**	Individual	7.92 E-01	4.40 E-04	18000	16.02	0.0001**
Side	1.15 E-01	5.79 E-03	20	49.18	0.0001**	Side	1.10 E-02	5.22 E-04	20	19.02	0.0001**
Ind*Side	1.64 E-01	1.17 E-04	14000	7.28	0.0001**	Ind*Side	4.94 E-02	2.74 E-05	18000	7.63	0.0001**
Measurement error	9.91 E-02	1.61 E-05	56080			Measurement error	8.49 E-05	2.12 E-06	40		
hairy-tailed bolo mouse (*Necromys lasiurus*)						amazonian red-sided opossum (*Monodelphis glirina*)					
Centroid size						**Centroid size**					
Effect	SS	MS	df	F	P	Effect	SS	MS	df	F	P
Individual	3.15 E+01	5.26 E-01	60	32.6	0.0001**	Individual	3.23 E+02	4.04 E+00	80	143.65	0.0001**
Side	5.10 E-02	5.16 E-02	1	3.2	0.078ns	Side	3.32 E-01	3.32 E-01	1	11.79	0.0009**
Ind*Side	9.85 E-01	1.61 E-02	61	3.10	0.0001**	Ind*Side	2.28 E+00	2.28 E-02	81	2.46	0.0001**
Measurement error	1.28 E+00	5.21 E-03	247			Measurement error	3.75 E+00	1.14 E-02	328		

Shape						Shape					
Effect	SS	MS	df	F	P	Effect	SS	MS	df	F	P
Individual	7.31 E-01	6.09 E-04	12 00	3. 53	0.00 01**	Individual	1.46 E+00	9.18 E-04	16 00	15. 83	0.00 01**
Side	4.59 E-02	2.29 E-03	20	13 .3 0	0.00 01**	Side	2.03 E-02	1.01 E-03	20	17. 53	0.00 01**
Ind*Side	2.10 E-01	1.72 E-04	12 20	8. 40	0.00 01**	Ind*Side	9.40 E-02	5,80 E-05	16 20	10. 32	0.00 01**
Measurement error	1.01 E-01	1.28 E-05	49 40			Measurement error	3.69 E-02	5.62 E-06	65 60		

Obs: SS: sum of squares, df: degrees of freedom, MS: mean squares, F: F-statistic, P: P-value – ns: not significant, **P< 0.01; *P< 0.05.

FA levels were not significantly different between sexes for all species (Table 6 -A). As such, data from both males and females was pooled for all subsequent analyses.

Table 6 - FA variation between sexes (A) and between fragment groups (B).

Species	A- Between sex			B - Between fragment´groups		
Rodentia	Females	Males	*P-value*	Small	Large	*P-value*
Proechimys longicaudatus	3.5±0.7	3.6±0.6	0.43ns	3.7±0.4	3.3±0.5	0.02*
Necromys lasiurus	3.4±0.8	3.6±0.7	0.28ns	3.5±0.8	3.5±0.7	0.96ns
Didelphimorphia	Females	Males	*P-value*	Small	Large	*P-value*
Marmosa demerarae	3.4±0.6	3.6±0.6	0.11ns	3.7±0.6	3.3±0.5	0.02*
Monodelphis glirina	3.5±0.7	3.5±0.9	0.68ns	3.4±0.8	3.5±0.8	0.43ns

***P< 0.01; *P< 0.05.*

The first two PCs used in the analysis explained 32.49% and 27.40%, respectively, of the total variation, accounting for 59.89% in total (see details in Figure 3). Landscape metric with greater contribution, significantly, in F1 (Factor loadings) was the Area. In the F2 the landscape metrics with greater contribution, not significantly, were the Isolation and Shape (see details in Table 7, Figure 3). Results of the correlations between the FA-species and the Landscape metrics are described in Figure 3, Table VII.

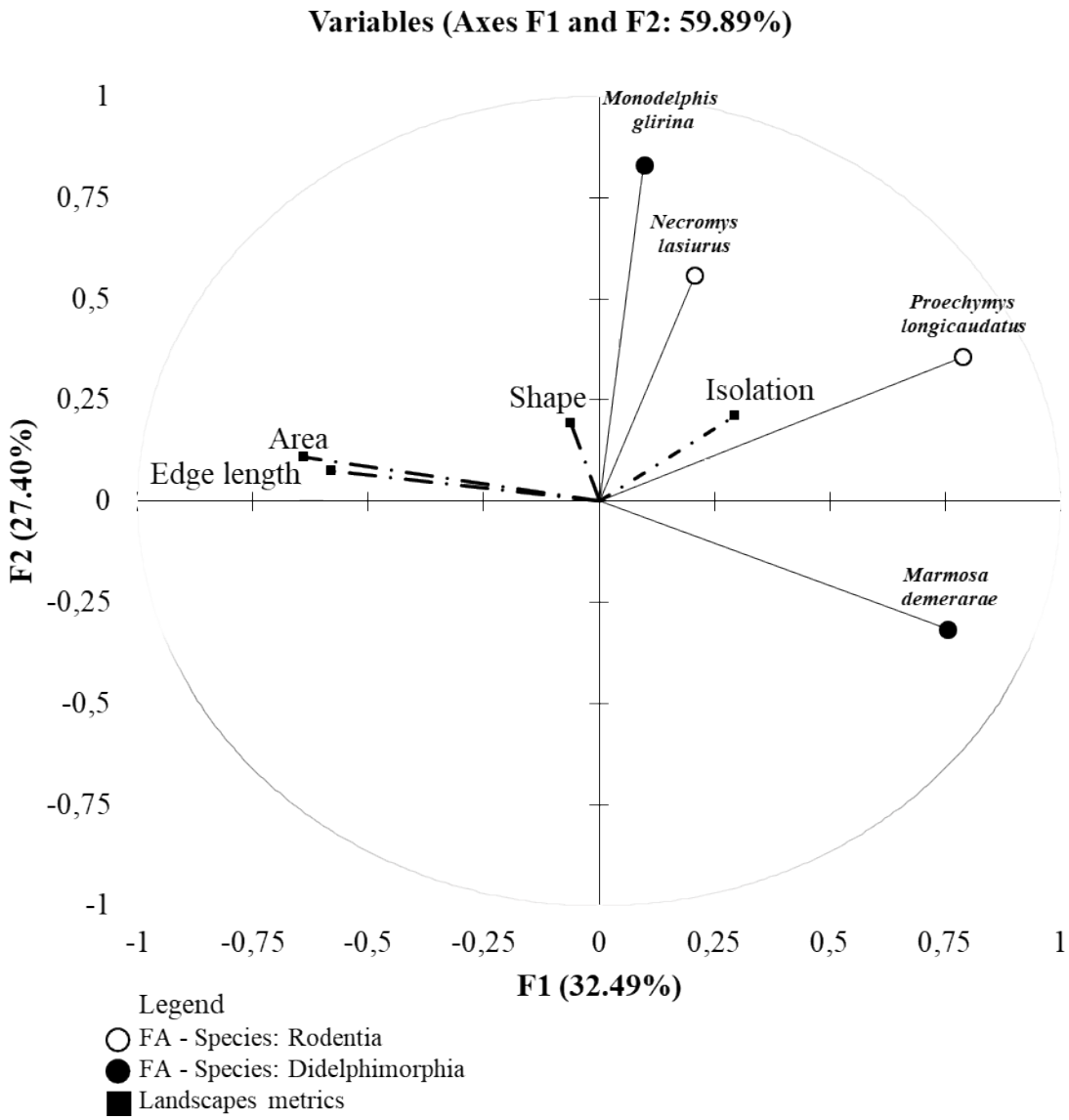

Figure 3 PCA (Principal Component Analysis) - ordination diagram in correlation biplot scaling with FA-species represented by circles and landscape metrics by squares.

Table 7 Principal components analysis (PCA) of Landscapes metrics associated with the average FA-species.

Landscape metrics	Factor loadings		Correlation Coefficient Matrix (Pearson)			
			FA - Rodentia		FA - Didelphimorphia	
	F1	F2	*Proechimys longincaudatus*	*Necromys lasiurus*	*Marmosa demerarae*	*Monodephis glirina*
Area	**-0.64**	0.110	**-0.614**	0.151	**-0.621**	-0.056
Edge length	-0.58	0.076	-0.372	0.045	**-0.534**	-0.012
Isolation	0.294	0.211	0.302	0.127	0.127	0.160
Shape	-0.06	0.193	0.110	0.057	-0.338	0.079

Obs. Values in bold are different from 0 with a significance level alpha=0.05.

Considering the different sized forest fragments, FA levels were significantly higher in small fragments for the larger study species, the long-tailed spiny rat (*Proechimys longicaudatus*) and the woolly mouse opossum (*Marmosa demerarae*) (Table 6 – B and Figure 4). On the contrary, the other two species hairy-tailed bolo mouse (*Necromys lasiurus*and) and amazonian red-sided opossum (*Monodelphis glirina*) revealed a complete overlap in the range of values of FA (Table 6 – B and Figure 4).

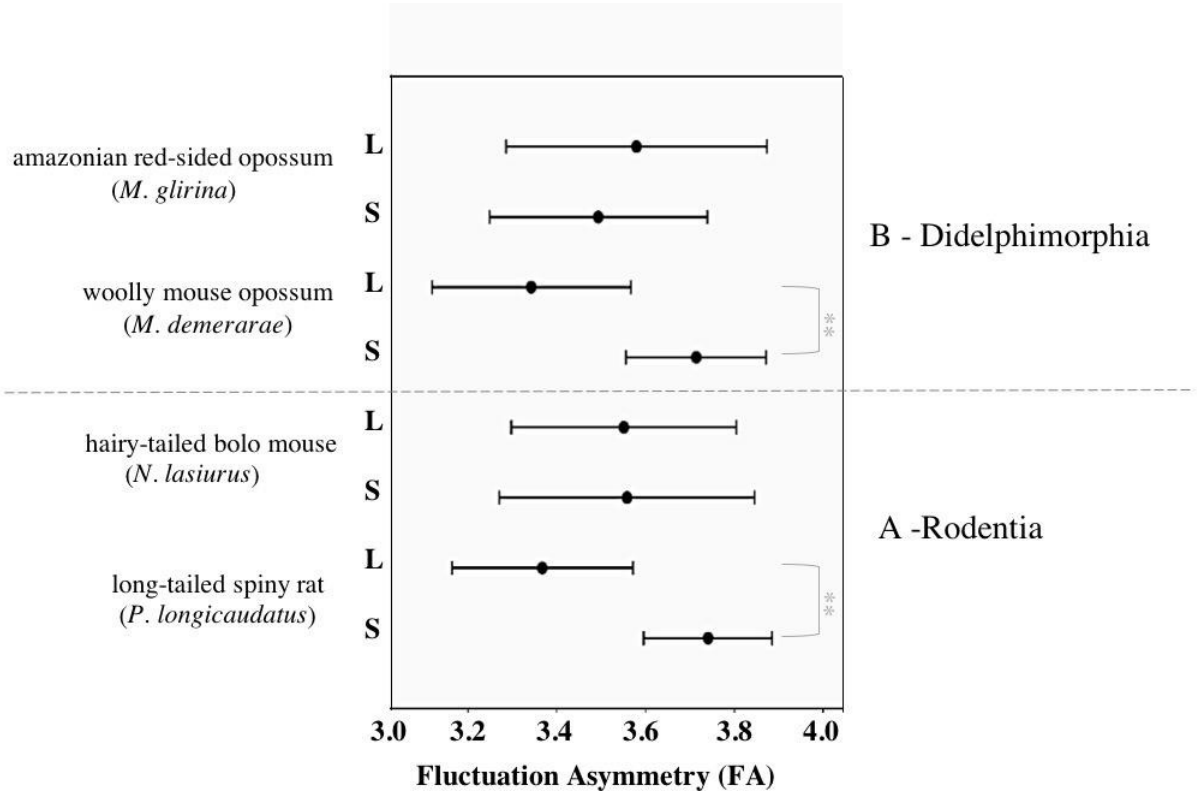

Figure 4 Variation in FA levels between groups of fragments, S - Small fragments, L - Large fragments; **P<0.005.

The isolation index of the fragments had a non-significant positive influence with FA for all species (long-tailed spiny rat (*Proechimys longicaudatus*), R^2 = 21%, p=0.45; hairy-tailed bolo mouse (*Necromys lasiurus*), R^2 = 15%, p=0.60; woolly mouse opossum (*Marmosa demerarae*), R^2 = 33%, p=0.31; amazonian red-sided opossum (*Monodelphis glirina*), R^2 = 16%, p=0.55) (see figure 5, Table 7).

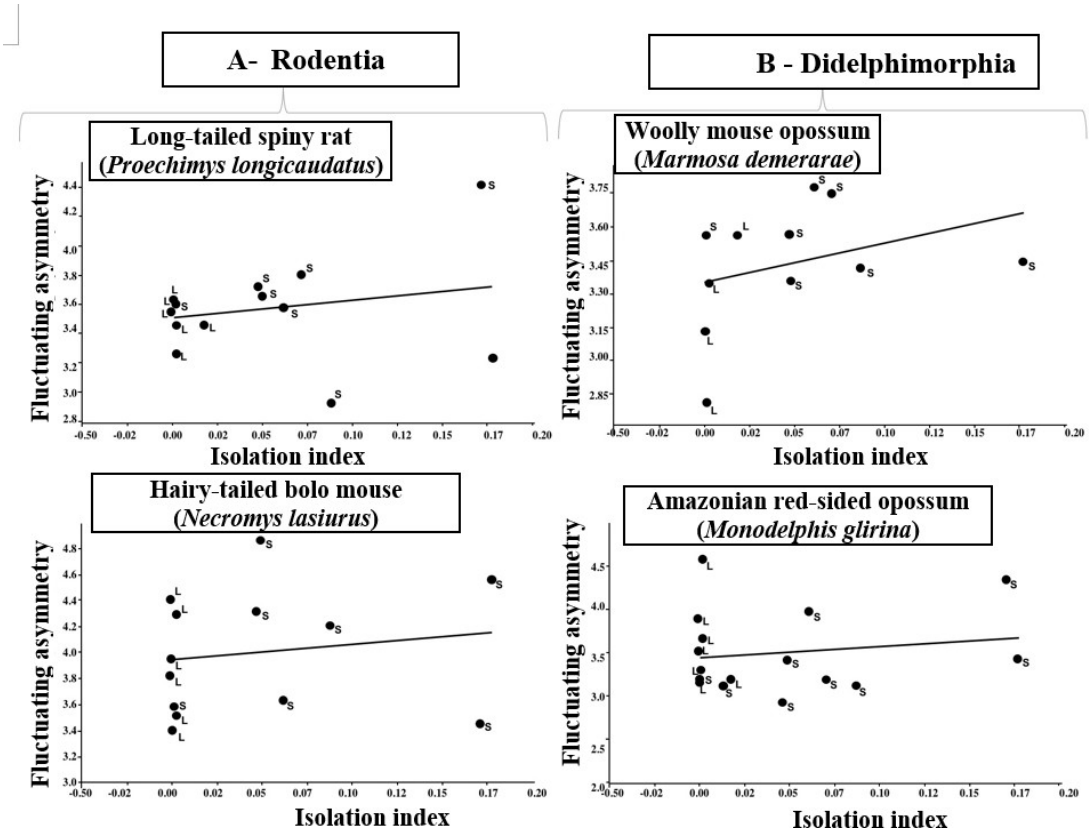

Figure 5 Linear regression showing the influence of fragment isolation levels on FA (S - Small fragments, L - Large fragments).

3.2.4. Discussion

In this study, we tested whether the environmental variables 'fragment area', ´edge length`, ´shape` and 'isolation' explained the observed variation in fluctuating asymmetry (FA) in four neotropical species of small mammals, the long-tailed spiny rat (*Proechimys longicaudatus*), the hairy-tailed bolo mouse (*Necromys lasiurus*), the woolly mouse opossum (*Marmosa demerarae*) and the amazonian red-sided opossum (*Monodelphis glirina*) inhabiting one of the most deforested and fragmented areas in southern Amazonia.

We did not find any pattern distinguishing Rodentia from Didelphimorphia; instead, we could only infer that some ecological characteristics shared by some of the species, regardless of their taxonomy, may have a more relevant role in the observed fluctuating asymmetry levels. For the two largest species, the long-tailed spiny rat (*Proechimys longicaudatus*) and the woolly mouse opossum (*Marmosa demerarae*), environmental stress during embryonic development seems to increase as the fragment area decreases, as reflected in the significantly augmented levels of FA observed in small fragments in comparison to

large fragments. Therefore, individuals from these species seem to be the most affected, with significant differences in FA explained by differential forest fragment size. Levels of FA were positively correlated with levels of fragment isolation, but non-significant for any of the studied species.

Anciães and Marini (2000) found a significant negative correlation between FA and increasing fragment area in a community of birds in the Brazilian tropical forest. The authors suggest that it may be due to genetic impoverishment or population size reduction, factors that increase as the suitable habitat size decreases. Large fragments, with a larger core area (more protected area, with more plant species, with a greater proportion of niches) present better biotic conditions, not leading to increased stress during developmental stages (Zimmerman and Bierregaard 1986, Stevens and Husband 1998).

The process of deforestation involves the creation of numerous clearings within the forest, dividing specific habitats and influencing landscape change, altering the size and availability of resources in the microhabitats that species uses (Laurance et al. 2009). One of the main impacts of this process involves the conversion of continuous forest to border habitats (Tinker et al. 1997; McGarigal et al. 2001). In forest fragments, the edge effect is more noticeable in small or narrow patches, which can be highly affected by external factors, therefore inflicting the most stressful conditions in the reduced forest core (Echeverria et al. 2008). FA levels of the smaller species, the hairy-tailed bolo mouse (*Necromys lasiurus*) and the amazonian red-sided opossum (*Monodelphis glirina*), showed no significant differences between different sized fragments, contrarily to the larger species, as referred above. According to Forman and Grodon (1986), species with larger body size suffer greater pressure than those with smaller size, since this trait is inversely related to population density. In this sense, there is still no consensus on the minimum viable forest area to maintain the community's functionality and viability as it may vary considerably according to the target/study species.

The lack of significant differences in FA levels between small and large fragment sizes concerning the hairy-tailed bolo mouse (*Necromys lasiurus*) may be related with the species' higher dispersion capabilities, exhibiting the largest home range among the studied species (see Table 3). *Necromys lasiurus* is a common small mammal, reaching high

population densities throughout the year, also found at great distances from the fragments' edges (Pires et al. 2010). Like for other study species, FA levels showed a positive (but not significant) correlation with fragment isolation. Even though it is classified as arboreal, the species makes more use of the ground (Leiner et al 2010) than *Marmosa demerarae* and is capable of dispersing for greater distances across the "matrix". *Marmosa demerarae* has a more reduced dispersion capacity in more isolated fragments in the absence of connecting trees. However, for any of the analysed species, isolation has not yet severely impacted levels of FA despite the detected positive correlation (Figure 3, 5).

FA levels in *Monodelphis glirina* also did not present significant differences between small and large fragments. This species generally has fairly small territories, is extremely territorial, and is one of the didelphid species less dependent of the arboreal vegetation (Nowak 1999). *Monodelphis glirina* is a ground dweller with preference for specific microhabitats where it can find shelter and food, with availability of branches and decomposing trunks, leaves, beetles, diplopods and animal excrements. The decrease of this microhabitat type leads the species to use different strategies, becoming opportunistic and more generalist in the search for food resources (Castilheiro and Santos-Filho 2013). One of them involves the use of the forest fragments' edges near the pasture matrix, where it finds a somewhat similar micro-habitat due to the presence of cattle feces that attract beetles (Castilheiro and Santos-Filho 2013). Also, at the edges, a higher exposure to wind increases the fall of trees and leaves (Laurence and Curran 2008), providing favourable conditions for the occurrence of the species.

Animal species that are more resilient to the effects of forest fragmentation generally share some characteristics related to the ecological requirements of each species that allow them to positively respond to edge formation (da Rosa et al., 2017), namely requiring less extensive core area and being "matrix" tolerant (Tocher et al.1997, Kalko 1998). Tocher et al. (1997) provides an example in which the persistence of frogs in forest fragments in central Amazonia is attributed to the fact that the species occupy a small area still being able to use the matrix among the fragments. Kalko (1998) reports that frugivorous bats that are able to forage in the matrix habitats may become more abundant in the fragments than in the continuous forest if the matrix as long as a large supply of food resources is available.

FA levels in *Monodelphis glirina* and *Necromys lasiurus* did not present significant variation among small and large fragments. Both are ground-dwelling species with high tolerance to habitat change (Pires et al. 2010, Castilheiro and Santos-Filho 2013), have a small body size, a lower dependence on trees, usually occur at high population densities, and have a more generalist diets (see Table 4). These common traits seem to, so far, have granted both species a greater resilience to environmental stress and its potential consequences during the developmental stage.

Although not all study species showed evidence of deleterious impacts of forest fragmentation regarding FA levels, we highlighted that for those who did (*Proechimys longicaudatus* and *Marmosa demerarae*), fragment size (area) seemed to be significantly more important than isolation factors in terms of developmental stress experienced by the populations. It is widely accepted that the inability or lower ability of individuals to move between isolated fragments can result in reduced gene flow and increased inbreeding, potentially leading to a significant reduction in the genetic variability of the meta-populations (Brito and Fernandez 2000, Brito 2009). This phenomenon can be compared with what occurs in island populations, where individuals are restricted to a discrete geographical area, surrounded by unfavorable habitat. The black-footed rock-wallabies inhabiting the Barrow Island, Western Australia, exhibit extremely low levels of genetic diversity which has led this population into inbreeding depression, with evidence of reduced fecundity and increased FA levels when compared with continental populations (Eldridge et al. 1999).

Considering that no data is available regarding the matrix characteristics in our study area, we can hypothesize that matrix quality may be an important factor to consider in future studies as it may provide some degree of permeability and connectivity between at least some fragments (Arroyo-Rodríguez et al., 2013; Sánchez-de-Jesus et al., 2016). Whether the matrix connecting the fragments exhibits a proportion of secondary forest and/or arboreal crops providing additional foraging habitat for some of the study species, is merely speculative. However, this would help to explain the non-significance of the effect of fragment isolation in observed FA levels.

Effects of environmental variables of fragments such as area, border (edge length), shape, isolation, and matrix quality on the community structure of species are reported by a

series of authors. Wilcox and Murphy (1985) and Keinath et al (2017) argue that large mammals are extremely dependent on large areas, are especially vulnerable to fragmentation due to their large body size and trophic needs, i.e., for the authors, fragmentation is the most serious threat to biological diversity and is the primary cause of the present extinction crisis. Laurance and Yensen (1991) and Rocha et al. (2018) proposed models to evaluate the ecological impacts of edge effects on natural habitat fragments surrounded by artificial matrix, model simulations revealed that for any edge-sensitive species and habitat type there exists a critical range of fragment sizes in which the impacts of edge effects increase almost exponentially, demonstrating the edge effect as a negative consequence of the fragmentation process. Ranta et al. (1998), Hill and Curran (2003) consider shape, size, central area, matrix quality and isolation of the fragments as variables that have important roles for the species conservation, suggesting that reforestation of sugar cane fields between the forest fragments would considerably increase the area of interior forest habitat and connectivity between fragments.

In general, variables that most explain the development′s stress present in the small mammals are related to the fragments′area, changes in area mean that all other landscape metrics also undergo changes, as an example, correlations between the area are positive and relatively high with the proportion of landscape, the edge and the central area, and negative with the isolation′s degree, this pattern may change depending on the matrix′s quality, i.e. the degree of connection between fragments.

The studied meta-populations continued to persist over the years under the effects of increasing fragmentation in southern Amazonia. Both rodents and didelphids appear to have plasticity to survive in more diverse environments and are somewhat resilient under stressful environmental conditions.

However, despite all possible environmental descriptors of forest fragments, changes in fragment area are always the most explanatory factor for observed oscillations in abundance, richness and equitability of small mammals' communities (Pardini 2004; Michalski and Peres 2007; Vieira et al. 2009). Our results support that fragment area clearly has a significant influence on FA levels, reinforcing that forest fragmentation impacts on

small mammal communities are not solely restricted to changes in number of individuals or species but also impacting individual specimens.

In this study, we highlighted the main landscape metrics showing the most significant contributions towards the levels of FA observed in forest fragments that have explained the stress in development experienced by populations. However, in the long term, the level of disturbance will ultimately cause an irreversible disequilibrium in the community structure of at least some of the small mammal species inhabiting the forest fragments.

3.2.4.1. Future prospects

Numerous collections of multiple species have already been carried out in the Amazon rainforest over the years. A time series, before and after the fragmentation process started, is available, not only of small mammals but also of large mammals and birds, many of them considered bioindicators of environmental quality. These collections are rarely used with the purpose of analysing morphological structures, using a geometric morphometric approach. Also, numerous specimens are killed while trying to cross the roads dividing the forest, being completely discarded after collection of a small tissue sample by environmental agencies. Even if only partially intact, lots of useful information is lost that could be useful towards a better understanding of this fragmentation dynamics.

Despite all limitations and challenges (Leung et al. 2003), measurement of FA levels has proven to be an effective biomonitoring tool for conservation biology, including of critically endangered species (Lens et al., 2002). Museum and research collections that house specimens (mandibles and/or skulls) collected during pre-fragmentation years constitute valuable repositories of information that can be compared with recently collected material from the forest fragmentation era. By analysing 50+ years old material, it would be possible to establish a baseline on what was the FA natural range under an undisturbed scenario of continuous forest. This way, it would be possible to measure the real impact of forest fragmentation along a time series, from a natural pristine forest setting to a current highly fragmented scenario. A major limitation for future studies involving wildlife relates to the fact that geometric morphometric techniques traditionally rely on invasive sampling methods, making use of morphological structures that implied the animal's death (e.g. skulls,

mandibles, teeth). However, if non-invasive methods start to emerge applied to ecological surveys, measuring fluctuating asymmetry would constitute a fast, reliable and inexpensive tool to asses environmental stress in mammals, particularly in the Neotropics, where such approaches clearly lack (Coda et al. 2017).

The specimens analysed in this study were collected in 2009 in Amazonian forest fragments that, in the meanwhile, have suffered further reductions in area despite all claims against deforestation. The 2019 massive fires throughout the Amazon forest further contributed to the acceleration of an already dramatic habitat loss and fragmentation scenario. The information retained in these collected specimens and their mandibles alerts for the need of a multidisciplinary approach to gather as much species-specific scientific information as possible in order to allow for more effective conservation measures in the Amazon rainforest. Even though the population decline effects may not be quantifiable in the short term after the fragmentation process starts, results presented herein show that the deleterious impact on the small mammal community is measurable at the individual level before the populations start to effectively decline.

3.2.5. Acknowledgments

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3.2.6. References

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4. Chapter 4

4.2. Effect of forest fragmentation on mandibular modularity and morphological integration of Neotropical small mammals in southern Amazonia*

*Data in this chapter will be submitted as a paper to PeerJ

Effect of forest fragmentation on mandibular modularity and morphological integration of Neotropical small mammals in southern Amazonia

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Abstract

Habitat loss and fragmentation are expected to be reflected in the habitat specialization of individuals facing such changes in their environment. One potential outcome of habitat fragmentation is the development of spatial structure in small mammals' populations, with the organisation of individuals with similar morphological and genetic characteristics into defined spatial units.

In this study, the main objective was to evaluate the effect of habitat fragmentation as a source of environmental stress on the mandible's bimodular organization and morphological integration in four Neotropical species of small mammals.

Study area is embedded in the forefront of the so-called 'arc of deforestation' in the Brazilian southern Amazonia. Specimens were collected between May and September 2009, from 13 forest fragments ranging from 4 to 1763 hectares. A total of 194 specimens were analysed, 78 rodents (*Phoechimys longicaudatus* and *Necromys lasiurus*) and 116 didelphids (*Marmosa demerarae* and *Monodelphis glinira*). Covariance matrices of the asymmetric component of shape variation were used to test the mandibles modularity following procedures. Principal Component Analysis (PCA) was performed for quantifying morphological integration in alveolar region and ascending ramus in mandibles using the distribution of eigenvalues.

Mainly, it was possible to demonstrate that for most study species, animals residing in small forest fragments, the bimodular mandibular structure was lost in comparison with those

animals living in large fragments, which exhibited the expected pattern of mandibular organisation in alveolar region and ascending ramus. In addition, the degree of modular integration was lower for both modules in small fragments. The exception involved the only arboreal species, the woolly mouse opossum, *Marmosa demerarae*, which exhibited no modular structure in both small and large fragments. Based on our results, it seems clear that some level of morphological disruption is already taking place as a result of the changed dynamics in a forest fragmentation context. In particular, species inhabiting small fragments are undergoing significant changes in their mandible's modularity and morphological integration that may result in unpredictable consequences for its structural functionality. These results unveil the need to further address such impacts of the environmental stress posed by forest fragmentation.

KeyWords: Amazon tropical forest, Fragmentation, Neotropical Rodentia, Neotropical Didelphimorphia

4.2.1. Introduction

Modularity and morphological integration are two closely related concepts at the intersection between evolution and development of organismal form referring to the tendency of different traits to jointly vary, in a coordinated manner, in a morphological structure or even a whole organism (Olson and Miller 1958, Klingenberg 2008, 2014, Goswami and Polly 2010). This inter-trait relationship can bias the direction and rate of morphological change, either facilitating or constraining evolution along certain dimensions of the morpho space (Porto et al., 2009). The study of morphological integration and modularity has witnessed a resurgence over recent years, partly because of this recognition that their patterns can profoundly affect how phenotypic changes occur and how new morphologies originate. Morphological integration is mostly inferred from data on covariation of multiple traits, implying that an evolutionary change in the morphology of one anatomical element is reflected in morphological changes in other elements (Smith 1996; Klingenberg 2008).

Integration is strongest if all variation is concentrated in a single dimension, indicating perfect correlation of all measurements, and is absent if variation is evenly distributed over all available dimensions (Klingenberg 2008, 2009, 2014). Modularity assumes a general property of biological systems to be organized into modules, units within which there is a high degree of integration but being relatively independent of other such

units (Klingenberg 2008). In the context of a morphometric analysis, these interactions will manifest as strong covariation among parts within modules and weak covariation between modules (Klingenberg 2008, 2009).

Depending on the processes responsible for integration and modularity, several levels of integration can be distinguished (developmental, functional, environmental and genetic (Klingenberg 2014). When traits originating from the same developmental precursor share the variations accumulated prior to or at the moment of partitioning of the precursor, morphological integration occurs as a result of these direct developmental interactions (Riska 1986), or variation can be transmitted between different precursors by inductive signalling (Jacobson and Sater 1988). Covariation of morphological traits derived from parallel variation in separate developmental pathways arises when some external factor (allelic or environmental variation) affects these pathways simultaneously (Klingenberg 2008).

The present study focuses on the mammalian mandible, a favored model system for studies of modularity and integration (Hall 2003, Klingenberg et al. 2003). The mandible consists of two primary units: the alveolar region (anterior part, supporting the teeth) and ascending ramus (articulating with the skull and providing surfaces for muscular fixation) (Klingenberg et al. 2003). These fundamental units in the mandible correspond to functional units according to their embryonic origin (Klingenberg et al. 2001b). Klingenberg et al. (2003) considers that the alveolar region and ascending ramus are separate developmental modules, but that the separation between them is not complete, so the changes in these modules must be measured by the degree of integration.

Some studies suggest that stress-induced variation can be accommodated by altering the organisms' integration (Badyaev 2005). For example, in island populations, integration can be altered at least in two different ways: on one hand, integration can increase because morphological variation in traits developing later during ontogeny is directed by stress- induced variation in traits that have developed earlier (Badyaev 2005); on the other hand, integration can decrease because inbreeding reduces genetic covariation (Phillips et al. 2001), which is a substantial component of morphological covariation arising from genetic linkage between loci and pleiotropic effects of a single locus (Klingenberg 2010).

Whether integration can be impacted by habitat fragmentation as an environmental stressor is still unknown. Forest fragmentation involves the transformation of a continuous forest into different sized patches, usually isolated, much like islands, linked by mostly unfavourable matrix habitat as a result of anthropogenic action.

Significant habitat alterations posed by fragmentation are expected to be reflected in the habitat specialization of individuals facing such changes in their environment. One potential outcome of fragmentation is the development of spatial structure in small mammals' populations, with the organization of individuals with similar morphological and genetic characteristics into defined spatial units (Ledevin and Millien 2013; Rogic et al. 2013).

The scenario we chose for this study involves a highly fragmented area of rainforest in southern Amazonia, in the so-called "arc of deforestation" (Barni et al. 2015).

Several species of small mammals have been resisting the forest fragmentation process (Laurance et al. 2011) but different species may respond at a different rate as a consequence of their ecological requirements and constraints. However, the consequences for the functionality and morphological variation due to environmental stress during development need to be further clarified (see Castilheiro et al. in prep2). Small mammals are considered good subjects to test hypothesis regarding population growth, migration, reproduction, and how the environment's complex physical structure affects the ecology of populations and communities at the landscape level (Barret and Peles 1999).

Geometric morphometrics is a valuable tool to evaluate deviations in symmetry or changes in the pattern of bimodularity (Teixeira et al. 2006; Jojic et al. 2012). However, studies assessing the impact of environmental stress on modularity and morphological integration are lacking. In this context, we analysed the asymmetric component of mandibular variation (i.e., deviations from expectedly symmetric morphological structures), considered an indicator of developmental instability (Neustupa and Nemcova 2018) by comparing covariation within- and between-modules (Jojic et al. 2012; Martín-Serra et al. 2014; Yalkovskaya et al. 2015; Quinto-Sánchez et al. 2018).

In this study, our main goal was to evaluate the effect of habitat fragmentation on the bimodular organization of the mandible (alveolar region and ascending ramus) using small mammals inhabiting a highly fragmented area as models. We evaluated if variation in the modularity and integration is correlated with the decrease in size of forest fragments by testing four different species living either in small or large forest patches.

4.2.2. Material and Methods

4.2.2.1. Study area and data collection

The study area is embedded in the forefront of the so-called 'arc of deforestation' in the Brazilian southern Amazonia, located in the municipality of Alta Floresta, Mato Grosso (see Figure 1). Specimens were collected between May and September 2009, from 13 forest fragments ranging from 4 to 1763 hectares (h). Fragments were grouped by size range (Small fragments: 4-7h and Large fragments: 106-1763h).

Mandibles (pairs of left and right hemimandibles) from a total of 194 adult specimens were analysed: 39 individuals of the long-tailed spiny rat (*Proechimys longicaudatus*), 39 of the hairy-tailed bolo mouse (*Necromys lasiurus*), 53 specimens of the woolly mouse opossum (*Marmosa demerarae*) and 63 specimens of the amazonian red-sided opossum (*Monodelphis glirina*) (see details in Figures 3).

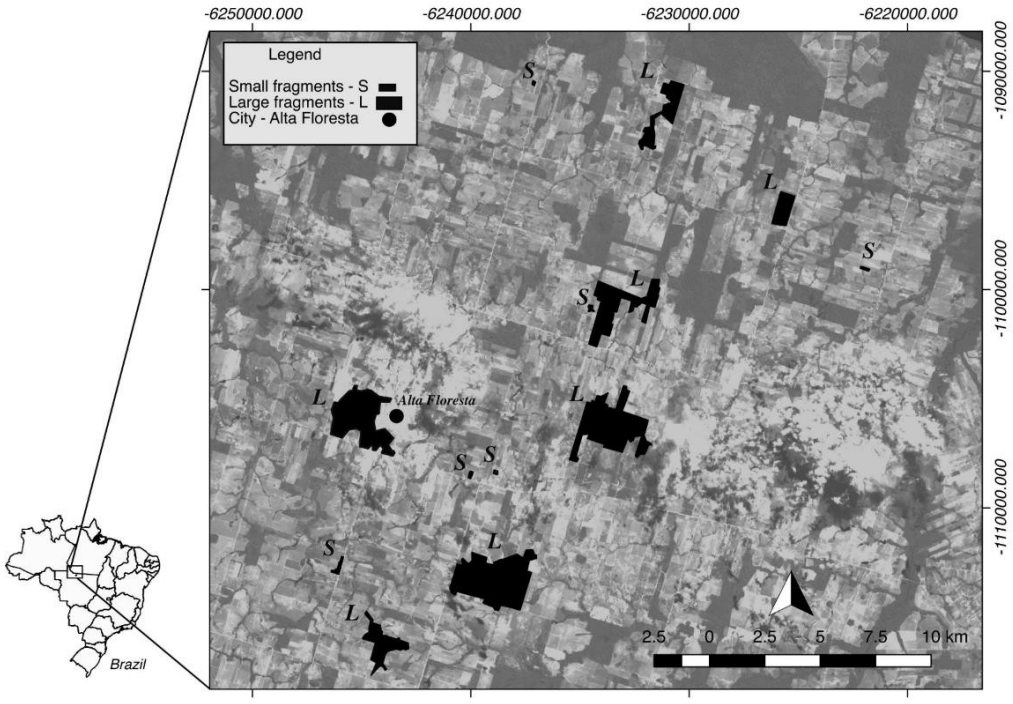

Figure 1 Map of forest fragments sampled in southern Amazonia, Alta Floresta, Brazil.

All analysed specimens originated from a collection held at the Mammalogy lab at the State University of Mato Grosso (UNEMAT, Brazil). Analysed mandibles were cleaned through exposure to a dermestid beetle colony (Muñoz-Muñoz et al. 2011; Franchini et al. 2016), separated into left and right hemimandibles and both photographed with a high resolution (20.1 megapixels) digital camera on the labial view. All photographs were taken using a copy-stand and under the same lighting conditions. Images of the left hemimandibles were reflected to their mirror images by assigning a negative sign to their x coordinates. A total of 12 anatomical landmarks located around the outline of the two-dimensional mandible photographs were digitised using TpsDig (Rohlf 2006), always by the same operator and in triplicate for error measurement (Figure 2, see Table 1 for a complete description of the exact location of the landmarks).

4.2.2.2. Data analyses

Prior to further analyses, mandible size variation was eliminated by scaling all specimens to unit centroid size, limiting analyses to variation on the asymmetric component of shape.

Covariation of the fluctuating asymmetry component of shape is exclusively due to the direct interactions between all the processes in place during individual embryonic development. Instead, trait variation among individuals also reflects other factors (Klingenberg et al. 2003). As such, covariance matrices of the asymmetric components of shape variation were calculated with MorphoJ version 1.06d and used to test the mandibles' modularity following Klingenberg (2009). After correction for allometric effects (please see Castilheiro et al., in prep1 for details), a Generalized Procrustes Analysis (GPA) and a Two-way Multivariate Analysis of Variance (MANOVA) were carried out. These sets of landmarks as the hypothesized modules, and alternative partitions were spatially contiguous. Mandibles were organized into two modules defined *a priori*, alveolar region and ascending ramus, corresponding to landmark sets 1 and 2, respectively. For both rodents, *P. longicaudatus* and

N. lasiurus, set 1 included the shape comprised by connecting landmarks 1-2-3-4-10-11-12-1, corresponding to the alveolar region and for set 2, landmarks 5-6-7-8-9, matching the ascending ramus. Concerning both didelphids, *M. demerarae* and *M. glirina*, set 1 included the shape comprised by landmarks 1-2-3-4-5-10-11-1 and set 2, landmarks 6-7-8-9-12 (see details in Table 1, Figure 2).

Table 1 Location of 12 anatomical landmarks in each hemi-mandible of the four small mammal species analysed, rodents and didelphids, respectively.

Rodentia (A)	
Landmark	Location
1	Most cranio-dorsal point of the mandibular symphysis that meets the posterior part of the incisor's alveolar margin
2	Point of maximum concavity between the incisor's alveolus and the tooth row
3	Cranialmost point of the tooth row's alveolar margin
4	Caudalmost point of the tooth row's alveolar margin
5	Tip of the coronoid process
6	Cranialmost point of the edge of the condyle's articular surface
7	Caudalmost point of the edge of the condyle's articular surface
8	Point of maximum concavity between the condyloid and the angular process
9	Tip of the angular process
10	Point of maximum concavity of the mandible's ventral margin
11	Point of maximum convexity of the dentary in the cranio-ventral part
12	Most cranio-ventral point of the mandibular symphysis that meets the anterior part of the incisor's alveolar margin
Didelphimorphia (B)	
Landmark	Location
1	Base of the lower first incisor
2	Base of the lower fourth incisor
3	Posterior base of the lower canine
4	Posterior base of the first molar
5	Posterior base of the fourth molar
6	Central point in the coronoid process
7	Endpoint of the caudal border of coronoid process
8	Point of inflection of the curve between the mandibular condyle and the caudal border of the coronoid process
9	Highest point at end of side of the mandibular condyle
10	Landmark 5 orthogonal projection on the ventral edge of the mandible
11	Landmark 4 orthogonal projection on the ventral edge of the mandible
12	Foramen's edge

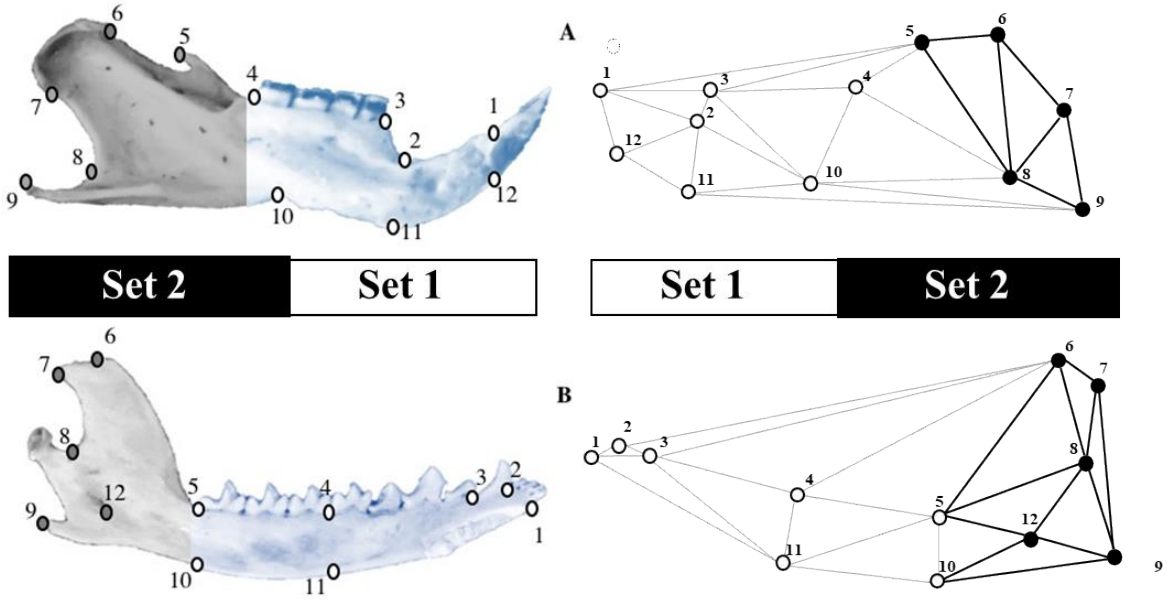

Figure 2 Two-modules organization in small mammals' mandibles considered a priori, according with Klingenberg and Navarro (2012) to the hypotheses of shared development and function. Set1: Alveolar region, Set2: Ascending ramus. A – Rodentia, B – Didelphimorphia. Numbers in the image (1-12) correspond to the digitised landmarks.

Traditionally, the RV coefficient (Escoufier 1973) has been mostly used when testing hypotheses of modularity in morphometric datasets (Adams 2016), however, RV-based procedures are sensitive to sample size and number of examined variables (modules). More recently, an alternative method, the covariance ratio (CR) has been proposed (Adams 2016), overcoming these limitations while exhibiting a higher statistical power when used to identify and quantify modular structure. CR compares covariation between-modules with covariation within-modules, ranging from zero to positive values: if the CR is high it reveals a high between-module covariation, a low within-module covariation or a combination of both (Adams 2016). This analysys was performed in R, *geomorph* package (Adams & Otárola-Castillo 2013; Adams et al. 2017). Histograms of CR coefficients were obtained from permutation tests of alternative partitions of the four small mammals' mandible, and the observed CR coefficient designated (Adams 2016).

For all four species, a principal component analysis (PCA) was performed for quantifying the Morphological Integration Index of the mandible for both the alveolar region and the ascending ramus, comparing small and large forest fragments, using the distribution of eigenvalues (Klingenberg and Zaklan 2000). The variance of eigenvalues measures integration by quantifying how much the variation is concentrated in just few dimensions or

spread across many directions of the shape space. If integration is absent, with equal amounts of variation in all directions of the shape space, variance will have its minimum value of 0. The index will reach its maximum if all variation is contained in a single dimension (complete integration) (see an example in Gómez et al. 2016). Because the covariance matrix is scaled in units of Procrustes distance, variances of eigenvalues are difficult to compare between different analyses. Here, the index incorporated the variance of eigenvalues scaled by the total variance and number of variables, ranging from 0 to a maximum of 1.

4.2.3. Results

4.2.3.1. Evaluation of an *a priori* hypothesis of a two modules mandible

A two-module organization of the mandible, composed by the alveolar region and the ascending ramus, was tested for all four study species and all but one (*M. demerarae*) complied with this pattern, however, only in large fragments. In small fragments, the covariance ratio (CR) in mandibles of the long-tailed spiny rat (*P. longicaudatus*) was CR=1.15, p=0.62, for the hairy-tailed bolo mouse (*N. lasiurus*) was CR=0.99, p=0.25, for the woolly mouse opossum (*M. demerarae*) was CR=1.01, p=0.24, and for the amazonian red- sided opossum (*M. glirina*), was CR=1.08, p=0.43, all not significant regarding levels of covariation between modules (see details in Figure 3). On the contrary, in large fragments, CR values were lower for both species (*P. longicaudatus* - CR=0.80, p=0.049, and *N. lasiurus* - CR=0.60, p=0.021), showing statistically significant differences between covariation between modules for both species (Figure 3).

In large fragments, the CR for mandibles of the woolly mouse opossums (*M. demerarae*) was CR=0.98, p=0.26, and for mandibles of Amazonian red-sided opossums (*M. glirina*), it was CR=0.78, p=0.042. Unexpectedly, it seems that the development in modules is lower or absent in the mandibles of woolly mouse opossums (*M. demerarae*). However, for Amazonian red-sided opossums (*M. glirina*), it seems the development in modules is present, as expected by the pattern for small mammals of the order Didelphimorphia (see details in Figure 3).

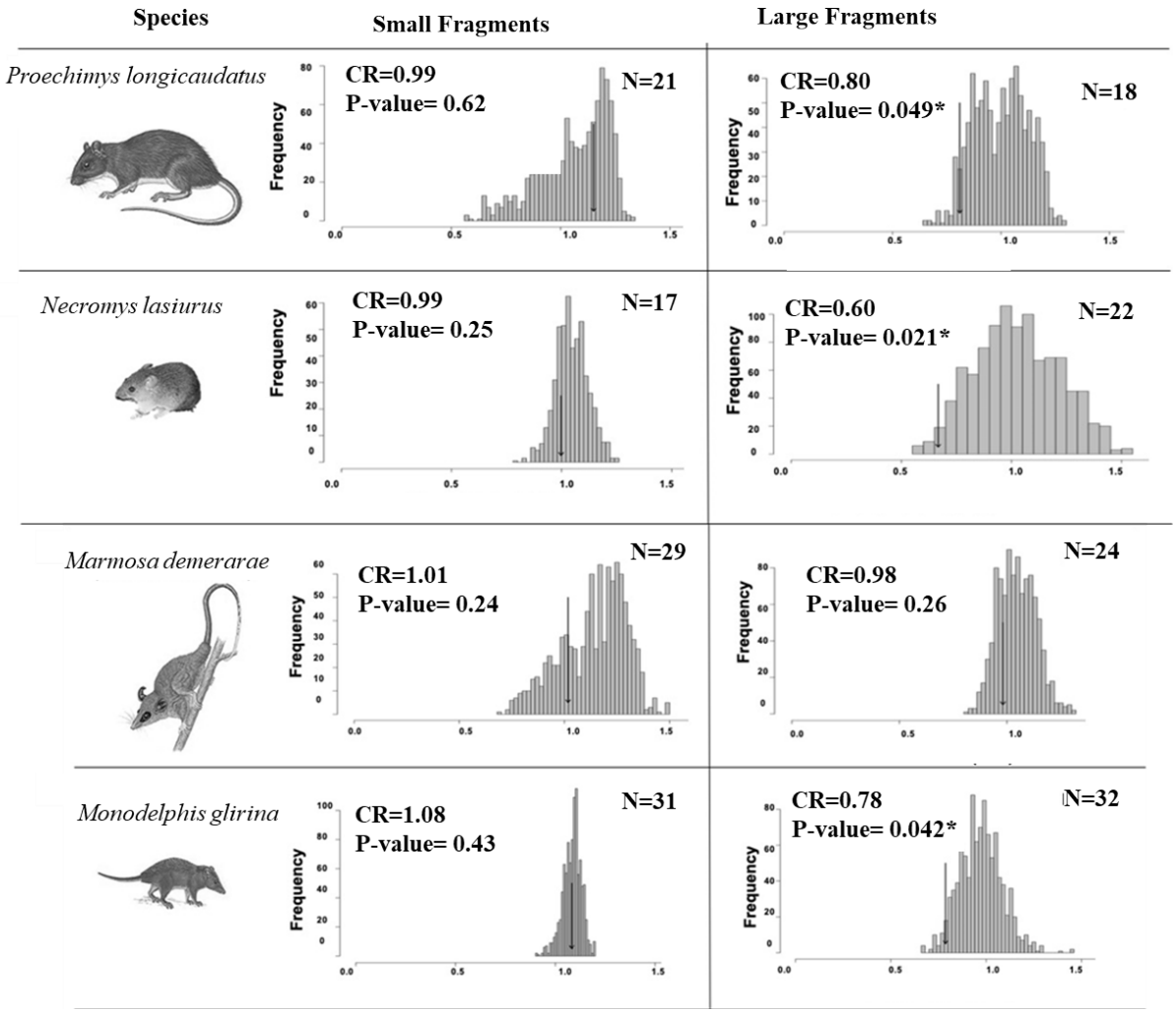

Figure 3 Analysis of modularity - evaluation of an a priori hypothesis of a two-modules organization of the mandible (Set1: alveolar region and Set2: ascending ramus) of two rodents and two didelphids considering the asymmetric component after correction for allometry. The values of CR coefficients (ratios comparing covariation between modules with covariation within modules) observed for the partition into alveolar region and ascending ramus are indicated with an arrow. Obs: species illustrations authored by Fiona A. Reid, N: Number of the individuals.

4.2.3.2. Morphological Integration Index

Morphological integration was lower for both mandibular modules (alveolar region and ascending ramus) in small fragments when compared to large fragments in both rodent species, *P. longicaudatus* and *N. lasiurus* (see details in Table 2). For the didelphid *M. glirina*, the same pattern emerged, however no differences were found between different sized fragments for the ascending ramus. In contrast with all other analysed species,

morphological integration for the woolly mouse opossum (*M. demerarae*) was similar between small and large fragments for both mandibular modules.

Table 2 Morphological Integration Index (MII) for the study rodents and didelphids.

Species	Alveolar region		Ascending ramus	
	Small fragments	Large fragments	Small fragments	Large fragments
Long-tailed spiny-rat (Proechimys longicaudatus)	0.225	0.473	0.186	0.434
Hairy-tailed bolo mouse (Necromys lasiurus)	0.124	0.323	0.223	0.349
Woolly mouse opossum (Marmosa demerarae)	0.143	0.145	0.084	0.074
Amazonian red-sided opossum (Monodelphis glirina)	0.259	0.490	0.114	0.133

4.2.4. Discussion

Numerous studies (Kozakiewicz 1993, Debinski and Holt 2000; Niebuhr et al. 2015) report the effects of area and isolation on the dynamics of forest fragmentation considering species' and community composition and distribution. In this study, we assess the impact of fragmentation through a novel approach addressing phenotypic change by exploring morphological integration and modularity patterns in small mammals' mandibles. Such changes were associated with differences in size of forest fragments under a deforestation scenario in the southern Amazonia by applying a geometric morphometric approach.

Different surveys using small mammals as study species have agreed that the mandible is divided into two main modules, the alveolar region and the ascending ramus (Klingenberg et al. 2003, Muñoz-Muñoz et al. 2011, Jojic et al. 2012). This bimodal organization of the mandible implies that both modules may follow somewhat independent evolutionary paths.

Among all study species, a bimodal organization of the mandible was confirmed for the three ground-dwelling (*P. longicaudatus*, *N. lasiurus* and *M. glirina*), but only for those populations living in large fragments (see Figure 3). In addition, for these same species, the degree of morphological integration was consistently lower in small fragments in opposition to large fragments and for both modules (alveolar region and ascending ramus) (Table 2). The only exception to this pattern considering both metrics involved the arboreal species *M.*

demerarae, which exhibited no mandibular modularity in either small or large fragments (see details in Figure 3). Likewise, in contrast with all other study species, the wooly mouse opossum exhibited very similar values of modular integration among the two modules (Table II).

Considering this bimodal organization as the normal and expected pattern, it seems that modularity in the mandible has been ruptured in small forest fragments, with similarly high values of covariance between tested modules, resulting in non-significance when the null hypothesis is tested (see Figure 3). This result highlights the fact that animals restricted to small forest fragments are subject to phenotypic alterations as a result of the environmental impact posed by fragmentation. Although no specific studies have addressed changes in modularity and morphological integration in a forest fragmentation context, it has been increasingly reported that environmental disturbance, either resultant of urbanization (Lazić et al 2015) or habitat destruction (Badyaev and Foresman 2004) has a significant impact in phenotypic variation of populations in the asymmetric component. Willmore et al. (2005) found higher levels of fluctuating asymmetry in macaque skulls associated with high levels of environmental variation. These authors' results coincide with those obtained in our study: the underlying processes that determine developmental stability are at least partly a result of environmental change and variation. Even if Amazonian species survive the process of deforestation, they may face alterations in modularity and morphological integration with putative impacts in the evolutionary pathways followed by the species' morphological structures.

The pattern observed in both rodent species, *P. longicaudatus* and *N. lasiurus* is also observed in the amazonian red-sided opossums, *M. glirina*. However, the woolly mouse opossum (*M. demerarae*) is the exception because the predicted modularity hypothesis was not confirmed for this species, neither in small fragments nor in large fragments. The covariance values between modules and within-modules are both high and similar, thus insignificant and not supportive of the tested hypothesis (Figure 4). In two previous studies, the wooly mouse opossum, *M. demerarae*, consistently showed different results when compared with the other study species as different aspects of morphological variation under a forest fragmentation scenario were addressed. Castilheiro et al. (in prep1; see details in chapter 2) found that females of this species have a high morphological similarity within- group of small fragments, and in Castilheiro et al. (in prep2; see details in chapter 3), the results showed significant differences in levels of fluctuating asymmetry between small and

large fragments, indicative of higher levels of stress during development. Possibly *M. demerarae* does not have mandible modules, which can be associated with arboreal habits, the arboreal habits can direct a different development from the other studied species, therefore the fragmentation responses are specific for each species. However, for the amazonian red-sided opossum *(M. glirina*), the modularity pattern was similar to that found for the study rodents, *P. longicaudatus* and *N. lasiurus*, with a high covariance value (statistically non-significant) in small forest fragments and a low covariance value (statistically significant) in large fragments, confirming the tested hypothesis of two-modules organization of the mandible. This pattern may also be associated with ecological characteristics of the species that potentially have an impact on development. Considering that the amazonian red-sided opossum (*M. glirina*) and the other study rodents have ground- dwelling habits, while the woolly mouse opossum (*M. demerarae*) is mostly arboreal, our results seem to indicate that, as a response to environmental changes posed by fragmentation, directional selection can also influence the modular structure, actively promoting a restructuring of genetic variation in the selected population and potentially facilitating the response to selection (Melo and Marroig 2015). Martín-Serra et al. (2014) compared the morphological integration magnitude in the appendicular skeleton of mammalian carnivores between specialised cursorial taxa (i.e. those whose forelimbs are primarily involved in locomotion) and non-cursorial species. Their results show that cursors have more integrated appendicular skeletons than non-cursors, concluding that natural selection influences modularity and morphological integration by increasing the degree of bone shape covariation in parallel to ecological specialisation.

Overall, most study species residing in small fragments exhibited alterations in modularity and morphological integration patterns in comparison with those inhabiting large fragments. Further studies are needed to better understand the results obtained for *M. demerarae*: samples from continuous forest areas would help to determine whether modularity and morphological integration patterns have also been ruptured in large fragments or if the species presents a different mandible organization under “natural” circumstances.

Our work opens space for two questions that need to be better answered: Do developmental stress and environmental variations in synergy determine the modularity at some level? Furthermore, what is the impact of modular disintegration and changes in the degree of integration on ecological specialisation for species living in small, isolated fragments?

4.2.5. Acknowledgments

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5. Chapter 5

5.2. Final Considerations

Final Considerations

Habitat loss and fragmentation have long been recognised as major drivers for biodiversity depletion and ecosystem degradation (Haddad et al. 2015). In recent years, several authors have investigated the deleterious effects of fragmentation on species richness and abundance in Neotropical small-mammal communities (Pardini et al. 2005; Rubio et al. 2014; Cerboncini et al. 2015; Gomez et al. 2015; Delciellos et al. 2015; Santos-Filho et al. 2016) as well as the effects on genetic diversity (Balkenhol et al. 2013). However, although many studies on mammals have focused on the response to forest fragmentation at the community level, few studies have evaluated whether habitat changes and anthropogenic pressure cause morphological alterations on target species (Manning and Chamberlain 1994; Wauters 1996; Teixeira et al. 2006). Considering that morphological alterations can affect the performance of individuals and, consequently, the structure of the biological communities associated with fragmented landscapes, the identification of changes at a phenotypic level may represent a useful indicator in conservation planning.

The main focus of this thesis was to investigate phenotypic effects of forest fragmentation on small mammals with different habitat use and ecological requirements, in southern Amazonia. Target species were two rodents with ground-dwelling habits and mostly omnivorous diets, the larger body size long-tailed spiny rat (*P. longicaudatus*), and the smaller hairy-tailed bolo mouse (*N. lasiurus*); and two didelphids, the woolly mouse opossum (*M. demerarae*), a larger didelphid with mostly arboreal habits and a preferential insectivorous diet but occasionally feeding on fruits, and the amazonian red-sided opossum (*M. glirina*), which is an insectivorous ground-dweller, and extremely territorial.

Geometric morphometrics was used as a tool to identify and compare morphological changes among species, at different levels.

We analysed mandible shape and size variations (Chapters 2 & 3). We tested whether fragment size and isolation can explain variations in fluctuating asymmetry. We also explored morphological integration and modularity patterns in the mandibles of individuals inhabiting a largely fragmented forest area (Chapter 4). We accomplished all this by comparing small and large forest patches as well as continuous forest areas, whenever samples were available. Understanding how small mammals explore a highly fragmented landscape and how fragmentation impacts these species provides results that can be applied to several other species.

Inhabiting small fragments has impacted all study species regarding the individual's mandible shape. Our results demonstrate that they undergo multiple morphological changes when the landscape configuration changed. In this context, this information should be better evaluated over time and considered when managing local biodiversity.

For the two largest species, the long-tailed spiny rat (*P. longicaudatus*) and the woolly mouse opossum (*M. demerarae*), significant differences in fluctuating asymmetry were explained by the difference in fragment size. This suggests that these small mammals exhibit morphological deviations from symmetry, most likely because of stress endured during development as a consequence of all the ecological alteration inherent to habitat fragmentation. Larger species tend to have larger home ranges which clearly is in conflict with the substantially reduced area in small forest fragments. Although small Amazonian mammals persist in the forest over the years, ecological characteristics, such as displacement capacity and habitat use patterns, can determine the vulnerability of a species to local extinctions due to habitat fragmentation.

Another important result to consider is that the resident animals in small fragments exhibit changes in the expected pattern of modularity and morphological integration, which can alter the individuals' structure and morphological functionality. These findings demonstrate that fragmentation can influence the pattern and degree of morphological integration by increasing the degree of bone-shape covariation. However, this result can be influenced by each species' degree of ecological specialisation (Martín-Serra et al. 2014).

Altogether, results demonstrate that geometric morphometrics can contribute to identify deleterious impacts of the disturbance caused by forest fragmentation. However, landscape planning should combine different sources of information, such as ecological and genetic data (Almeida et al. 2005). This combination will be extremely important for biodiversity conservation and will allow for an evaluation of the future accuracy of disturbance caused by habitat fragmentation.

5.2.1. Study limitations and constraints

Geometric morphometric studies based on morphological structures of animals pose a number of challenges and limitations sometimes difficult to overcome. Sample size in most studies involving animal structures is constrained by the total number of specimens available rather than an optimal number resultant of an *a priori* decision on sample size.

The major constraints of this study lied on the limited sample size of the study species. Indeed, this resulted in the exclusion of data from other small mammal species and/or other forest fragments because the number of available samples was too low. Also, when only very few fragments were sampled for a particular species, or the number of fragments sampled (small versus large) was very unbalanced, data was also disregarded. Additionally, morphological structures, particularly bone structures like those here used, are sometimes broken, even if partially, resulting in a further reduction in sample size. This is particularly troublesome when analyses involve bilateral structures, such as the assessment of fluctuating asymmetry in mandibles. Also, there is another issue to consider: if study species exhibit sexual dimorphism (therefore requiring separating the datasets in further analyses) sample size requirements become even more limiting.

Considering that numerous factors can affect variation in morphological structures, namely the mandible (e.g. phylogenetic history, recent adaptations to local environments, diet, habitat, etc.), a robust sample size is key to achieve solid conclusions. Despite these limitations, with the analysed dataset, it was possible to find consistent patterns across most study species.

Sample size and sample quality also constrained the overall study. It would have been very interesting to address questions related with genetic diversity within and between forest fragments and continuous areas and how those results correlated with those obtained for the morphometric component of the study. However, in landscape genetics studies, sample size and sample distribution are also a highly limiting factor in the pursuit of robust results and conclusions. The available tissue samples for a potential genetic diversity study failed to comply with the minimum requirements to reach solid conclusions within the scope of this thesis.

For future studies, in a forest fragmentation scenario and framework, it is critical that the original sampling design takes such factors into consideration: sexual dimorphism (if it is previously known), number and traits of the forest fragments to sample (e.g. area, shape, isolation), minimum number of samples per fragment, and suitable preservation of specimens/samples.

Nonetheless, despite all limitations, it is important to understand which analyses are more limited by sample size in order not to waist the potential of so much biological material enclosed, for example, in museum biological collections worldwide.

5.2.2. Impact for conservation of small mammals

Small mammals with ground-dwelling habits living in fragments of the Amazonian forest probably have higher dispersion rates, being able to leave the core of the fragment and forage in the pasture matrix (Ferraz et al. 2003). This behavior probably results in these species being more sensitive to morphological changes due to the habitat loss and forest fragmentation effects, as they are exposed to a higher mortality risk, more exposed to predation, dehydration, lower food availability, higher environmental stress and adverse effects that must be amplified in pasture between favourable habitats (Narayan and Williams 2016, Fahrig 2002).

In our study, smaller fragments were shown to be strongly associated with morphological changes in all addressed aspects (changes in modularity and morphological integration, fluctuating asymmetry and shape variation), showing the species' sensitivity to habitat reduction. Additionally, larger forest fragments suffer less micro-climatic and physical changes caused by the fragmentation process (Magnago et al. 2015). In a larger area, with higher habitat diversity and lower susceptibility to the edge effect, the environmental needs necessary for the species' persistence and reproduction are supported, resulting in lower impacts on morphology (Keinath et al. 2016).

Importance of fragment isolation was evaluated as a landscape metric in this fragmentation dynamics, assessing whether small mammals exhibited higher levels of fluctuating asymmetry as a consequence of developmental stress when inhabiting more isolated fragments. The obtained relationship was positive but non-significant. However, the most isolated fragments mostly overlapped with the smaller fragments, somehow mimicking the effect of fragment size. The dispersion of these species is favored in landscapes with lower isolation between-fragments, consequently lower morphological changes tend to happen (Laurance 2008). In fragments at lower distance from each other, dispersion is facilitated (Laurance 2008). Consequently, the need for between-fragments dispersion through the non-forest matrix is lower, reducing the exposure of species to threat factors (Pires et al. 2002).

On a local scale, fragment metrics consider both effects of habitat loss and changes in configuration resulting from the fragmentation process (Wilson et al. 2016). The amount of available habitat is an important characteristic for the persistence of small mammals' in fragmented landscapes and, when combined with the patches configuration (affecting

dispersion and connectivity of fragments), can be fundamental for the flow of individuals among fragments (Brad et al. 2011).

Fragment size seemed to be more important for larger species, such as *Marmosa demerarae* and *Proechimys longicaudatus*, since they both showed significant differences in fluctuating asymmetry when comparing small and large fragments. Larger areas have greater habitat availability and are less susceptible to the fragmentation effects, with a higher species number compared to smaller areas (Mullu 2016). Conservation measures for these species should give priority to increase the available forest area as well as aiming to reduce the effective isolation among the smaller remnant fragments (McAlpine et al. 2006).

As a very diverse group, the influence of landscape features in small mammal species can be almost species-specific, reflecting the natural history and behavior of each species (Michel et al. 2006). However, conservation measures should address the overall community and not just considering small mammals. In this context, maximum knowledge on multiple species is key to address the needs of as many species as possible. Although the conservation strategies used for the species have proven to be useful, when applied in isolation, they have flaws. Thus, it is necessary to adopt methods that involve several of these strategies in an integrated manner (Redford et al. 2011).

A landscape containing large Conservation Units interconnected by forest corridors and inserted in a matrix of agroforestry systems is perhaps a utopia in the current conjecture (Ramiadantsoa et al. 2015). But this may be the best alternative for the biodiversity conservation and ecological processes, especially if combined with environmental education actions that seek to include local communities in conservation programs (Ardoin et al. 2020).

Here we confirmed that the studied species respond differently to habitat loss and fragmentation. Studies aiming to unravel these effects on different animal groups should conduct species-specific analyses, since species from the same taxonomic group can respond differently to the same changes in the environment. In addition, efforts should be focused to construct general models that consider these different species' responses. Finally, we demonstrated how important it is to combine information at different morphological and landscape scales to unravel how species can persist in highly disturbed and fragmented landscapes.

5.2.3. Challenges for the study of the impact of forest fragmentation on small mammals in the Amazon rainforest

Addressing biological questions in the largest rainforest in the world, although highly exciting and appealing from a scientific point of view, presents tremendous challenges, of numerous orders. As such, all datasets available (particularly those resultant of past expeditions) are extremely valuable and should be explored to the maximum under different perspectives. Among the major challenges, it is possible to highlight the following:

i) Difficulties in logistics - one of the great challenges for the studies carried out in the Amazon forest is the logistics for field work and data collection. Extensive areas must be covered between sites, where access is usually very difficult. Projects carried out in this region usually have high costs and low rates of successful captures. To mitigate these difficulties, it is necessary to establish cooperation between different partners and local stakeholders.

ii) Difficulties in obtaining samples - one of the requirements of the geometric morphometrics methodology (as well as landscape genetics) is to obtain a robust sampling. However, in addition to logistic difficulties, sampling success may also depend on the target species, their size and abundance, making these sampling campaigns time consuming and dependent of numerous human resources.

iii) Difficulties in accessing larger fragments - larger fragments are generally located in private areas of the Amazon forest, within large farms and ranches. Most farmers restrict (or prohibit) entry in these locations. Common reasons for such restrictions also include conflicts with local and indigenous populations.

5.2.4. Future research

The achievements of this thesis can be extended in several directions. New questions and lines of research can be followed, warranting further investigation. We here demonstrated how geometric morphometrics can be used as a valuable tool contributing to identify morphological changes in individuals inhabiting a highly fragmented area in Amazonia. Methodologically, these same procedures can be applied to any other fragmented landscapes worldwide, addressing similar questions while studying other animal groups.

- ✓ The present study has produced results that explain how species of small mammals (species captured in small and large fragments) undergo morphological changes due to the fragmentation process. However, small mammals have different migration rates that vary according to their foraging and breeding habits. Evaluating how species move between fragments is essential for understanding which individuals are connected in metapopulations and which are isolated. GPS tracking a number of specimens of different small mammal species would provide additional insights on their dispersal abilities and putative consequences for the species maintenance in the medium/long term under a forest fragmentation context.

- ✓ We have also shown that larger species suffer higher developmental stress in small fragments, as well as changes in the morphological integration pattern. This could also indicate ecological specialisations of some species that should be addressed in the future in the search for those that are the most at risk of local extinction in contrast with others that could reveal higher levels of plasticity and higher resistance to the effects of fragmentation.

- ✓ Although we concluded that large fragments represent important repositories of more "natural" levels of morphologic diversity, it is important to highlight the future conservation projects need to consider that preserving small forest fragments is also paramount. Not only small fragments may harbor a higher number of rare and endemic species (Fahrig 2017) but they must also be considered key conservation elements as stepping-stones between other fragments, connecting them and reducing isolation between (meta)populations. This role of small fragments should then be further addressed in future studies.

- ✓ Museum and research collections of mandibles, skulls and other skeletal elements collected during pre-fragmentation constitute valuable repositories of information that can be compared with recently collected material. By analysing material that is over 50 years old, it would be possible to establish a baseline for the natural range of fluctuating asymmetry under an undisturbed scenario of continuous forest. Thus, it would be possible to measure the real impact of forest fragmentation along a time series, from a natural pristine forest setting to a current highly fragmented scenario.

5.2.5. References

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6. Supplementary Material

Table 1 -Description of total samples (mandibles) distributed by groups of fragments and continuous areas.

Sites	Area (ha)	Rodentia				Didelphimorphia			
		Proechimys longicaudatus		*Necromys lasiurus*		*Marmosa demerarae*		*Monodelphis glirina*	
		Males	Females	Males	Females	Males	Females	Males	Females
Small1	5	3	3	4	2	5	15	4	2
Small2	7	2	2	2	1	4	7	1	2
Small3	4	3	3	3	2	3	5	5	6
Small4	5	-	-	3	2	-	-	-	-
Small5	7	2	2	-	-	-	-	-	-
Small 6	15	2	2	3	1	3	6	10	7
Small 7	26	-	-	3	1	4	6	4	3
Small 8	5	3	4	-	-	-	-	-	-
Small 9	16	3	4	2	1	3	6	2	4
Subtotal		18	20	20	10	22	45	26	24
Large1	189	5	6	-	-	4	4	4	1
Large2	212	3	4	7	3	3	2	-	-
Large3	106	4	6	10	6	2	5	8	3
Large4	141	2	2	-	-	-	-	-	-
Large5	787	-	-	3	2	-	-	4	2
Large6	900	-	-	-	-	2	2	-	-
Large 7	123	-	-	-	-	-	-	3	2
Large 8	216	-	-	-	-	-	-	2	3
Subtotal		14	18	20	11	11	13	21	11
Continuous area1	30000	3	2	-	-	5	3	-	-
Continuous area2	30000	3	2	-	-	11	4	-	-
Subtotal		7	4	-	-	12	6	-	-
Total		39	42	40	21	45	64	47	35
		81		61		109		82	